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(54) Title: **EXPRESSION OF EUKARYOTIC PEPTIDES IN PLANT PLASTIDS**
 (54) Titre: **EXPRESSION DE PEPTIDES EUKARYOTES DANS LES PLASTES DE VEGETAUX**

(57) Abstract

Constructs and methods are provided for expressing peptides derived from eukaryotic organisms in plant plastids. Constructs have a promoter functional in a plant plastid, a DNA sequence encoding a peptide derived from an eukaryotic organism and a transcription termination region. Other elements include a selectable marker for selection of plant cells comprising a plastid expressing the marker and DNA regions of homology to the genome of the plastid and optionally a ribosome binding site joined to the promoter. By methods using such constructs high levels of eukaryotic peptides, such as mammalian proteins, are produced in a plant cell by growing plant cells under conditions whereby the DNA encoding sequences are expressed to produce eukaryotic peptide in said plastid.

(57) Abrégé

L'invention concerne des constructions et des procédés permettant d'exprimer des peptides dérivés d'organismes eukariotes dans les plastes de végétaux. Ces constructions possèdent un promoteur fonctionnel dans un plaste végétal, une séquence d'ADN codant pour un peptide dérivé d'un organisme eukariote, et une région de terminaison de transcription. D'autres éléments comprennent un marqueur sélectionnable, destiné à sélectionner des cellules végétales renfermant un plaste qui exprime le marqueur, des régions d'homologie d'ADN avec le génome du plaste, et éventuellement un site de liaison de ribosome relié au promoteur. Grâce aux méthodes utilisant ces constructions, des niveaux élevés de peptides eukariotes, tels que des protéines de mammifère, sont produits dans une cellule végétale par croissance de cellules végétales, dans conditions où les séquences de codage d'ADN sont exprimées de façon à produire un peptide eukaryote dans ledit plaste.

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(54) Title: EXPRESSION OF EUKARYOTIC PEPTIDES IN PLANT PLASTIDS			
(57) Abstract			
<p>Constructs and methods are provided for expressing peptides derived from eukaryotic organisms in plant plastids. Constructs have a promoter functional in a plant plastid, a DNA sequence encoding a peptide derived from an eukaryotic organism and a transcription termination region. Other elements include a selectable marker for selection of plant cells comprising a plastid expressing the marker and DNA regions of homology to the genome of the plastid and optionally a ribosome binding site joined to the promoter. By methods using such constructs high levels of eukaryotic peptides, such as mammalian proteins, are produced in a plant cell by growing plant cells under conditions whereby the DNA encoding sequences are expressed to produce eukaryotic peptide in said plastid.</p>			

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Description

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**EXPRESSION OF EUKARYOTIC PEPTIDES IN PLANT
PLASTIDS**

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INTRODUCTION

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Technical Field

This invention relates to the application of genetic engineering techniques to
10 plants. Specifically, the invention relates to compositions and methods for enhancing
expression of proteins in plant plastids.

20

Background

The plastids of higher plants are an attractive target for genetic engineering. Plant
25 plastids (chloroplasts, amyloplasts, elaioplasts, etioplasts, chromoplasts, etc.) are the major
biosynthetic centers that, in addition to photosynthesis, are responsible for production of
industrially important compounds such as amino acids, complex carbohydrates, fatty acids,
and pigments. Plastids are derived from a common precursor known as a proplastid and
30 thus the plastids present in a given plant species all have the same genetic content. Plant
cells contain 500-10,000 copies of a small 120-160 kilobase circular genome, each
molecule of which has a large (approximately 25kb) inverted repeat. Thus, it is possible to
35 engineer plant cells to contain up to 20,000 copies of a particular gene of interest which
potentially can result in very high levels of foreign gene expression. In addition, plastids
of most plants are maternally inherited. Consequently, unlike heterologous genes
40 expressed in the nucleus, heterologous genes expressed in plastids are not pollen
disseminated, therefore, a trait introduced into a plant plastid will not be transmitted to
wild-type relatives.

There remains a need for improved regulatory elements for expression of genes in a
45 plant plastid. To date, the expression signals used routinely for plastid transgene
30 expression derive from endogenous plastid genes. The plastid expression signals are
typically derived from promoter regions of highly expressed plastid genes such as the
promoter regions from the 16S ribosomal RNA operon (*Prrn*), *psbA* gene (*PpsbA*) or the
50 *rbcL* gene (*PrbcL*). The *psbA* and *rbcL* genes are highly transcribed, but their translation is

5 controlled by tissue-specific and light-regulated factors which limits their usefulness. In
the case of *Prm*, a synthetic ribosome binding site (RBS) patterned after the plastid *rbcL*
gene leader has been typically used to direct translation. However, this *Prm*/RBS is
translated inefficiently due to poor ribosome binding.

10 5 Plastids of higher plants present an attractive target for genetic engineering. As
mentioned above, plastids of higher plants are maternally inherited. This offers an
advantage for genetic engineering of plants for tolerance or resistance to natural or
15 chemical conditions, such as herbicide tolerance, as these traits will not be transmitted to
wild-type relatives. In addition, the high level of foreign gene expression is attractive for
20 10 engineered traits such as the production of pharmaceutically important proteins.

25 Expression of nucleic acid sequences encoding for enzymes providing for herbicide
tolerance as well as pharmaceutical proteins from plant plastid genome offers an attractive
alternative to expression from the plant nuclear genome.

25 15 **SUMMARY OF THE INVENTION**

30 The present invention provides nucleic acid sequences useful in enhancing
expression of a wide variety of genes, both eukaryotic and prokaryotic, in plant plastids.
35 Furthermore, plastid expression constructs are provided which are useful for genetic
engineering of plant cells and which provide for enhanced expression of the EPSP
synthase protein or the hGH protein in plant cell plastids. The transformed plastids
should be metabolically active plastids, and are preferably maintained at a high copy
40 20 number in the plant tissue of interest, most preferably the chloroplasts found in green plant
tissues, such as leaves or cotyledons.

45 25 The plastid expression constructs for use in this invention generally include a
plastid promoter region capable of providing for enhanced expression of a DNA sequence,
a DNA sequence encoding an EPSPS protein or human growth hormone (hGH), and a
transcription termination region capable of terminating transcription in a plant plastid.

50 30 The plastid promoter region of the present invention is preferably linked to a
ribosome binding site which provides for enhanced translation of mRNA transcripts in a
plant plastid.

55 The plastid expression construct of this invention is preferably linked to a construct
having a DNA sequence encoding a selectable marker which can be expressed in a plant

5 plastid. Expression of the selectable marker allows the identification of plant cells comprising a plastid expressing the marker.

10 In a preferred embodiment, vectors for transfer of the construct into a plant cell include means for inserting the expression and selection constructs into the plastid genome. The vectors preferably comprise regions of homology to the target plastid genome which flank the constructs.

15 The constructs of the present invention preferably comprise a promoter sequence linked to a ribosome binding site capable of enhancing the translation of mRNA transcripts in the plant plastid. The ribosome binding site is preferably derived from the T7 20 bacteriophage gene 10 leader sequence.

25 Of particular interest in the present invention is the high level of expression of nucleic acid sequences in plant plastids. Of particular interest is the high level expression 30 of nucleic acid sequences encoding for enzymes involved in herbicide tolerance and encoding for pharmaceutical proteins.

35 The constructs of the present invention preferably comprise a DNA sequence 20 encoding 5-Enolpyruvylshikimate-3-phosphate synthase (USPN 5,633,435, the entirety of which is incorporated herein by reference), nitrilase, phytoene desaturase, aprotinin or a 30 DNA sequence encoding human growth hormone (USPN 5,424,199, the entirety of which is incorporated herein by reference).

35 Plant cell plastids containing the constructs are also contemplated in the invention, as are plants, plant seeds, plant cells or progeny thereof containing plastids comprising the 40 construct.

45 The present invention also includes methods for enhanced expression of DNA sequences in plant plastids.

50 The invention also includes a method for the enhanced expression of an enzyme 25 encoding hGH in plastids of the plant cell.

55 The present invention further includes methods for obtaining a protein expressed 30 from a plant cell, including a plastid, having a non-methionine N-terminus. In addition, plant cells and plastids which include non-methionine N-terminus proteins are 40 contemplated.

55 Thus, the present invention relates to a chimeric gene containing a coding sequence 35 of a pharmaceutical protein, a plant plastid expression vector containing a promoter operably linked to a T7 Bacteriophage Polymerase gene 10 ribosome binding site capable

5 of enhanced expression in a plant plastid operably linked to a herbicide tolerance or pharmaceutical coding gene, a plant transformation vector having inserted therein a
10 herbicide tolerance or pharmaceutical coding gene expressed from a plastid promoter linked to a T7 Bacteriophage Polymerase gene 10 ribosome binding site , plant cells
15 transformed using such vectors and plants regenerated therefrom which exhibit a substantial degree of expression of nucleic acid sequences and proteins and methods for producing such plants and such plants.

15

10 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows the nucleotide sequence of the G10L ribosome binding site.

20 Figure 2 provides an amino acid sequence encoding for aprotinin.

Figure 3 provides the results of RP-HPLC analysis for characterization of hGH protein expressed in the plastid. Peak I (tallest peak) indicates the expected retention time
25 for properly folded, native 22 kDa GP2000.

15 Figure 4 provides an electrospray ionization mass spectrometry (MS) analysis using a Micromass Q-Tof electrospray time-of-flight mass spectrometer. In particular, a series of ions corresponding to the specie(s) present in the sample with varying numbers of
30 protons attached is provided. The axes of the spectrum are intensity versus mass-to-charge
20 ratio of the specie(s) present.

35 Figure 5 provides a graphic representation of the bioactivity of hGH expressed from a plant plastid. The samples represented on the graph are bovine prolactin (bPL),
hGH expressed from *E. coli* (Ala-hGH), and a null transgenic spiked with bovine prolactin
30 (SPFF Null Spike) as positive controls, a null transgenic (SPFF Null) as a negative control,
25 and transgenic samples from a sepharose column (SPFF Sample, SPFF Sample) and a
40 transgenic sample eluted from the sepharose column at pH3.5 (SPFF pH3.5 Eln).

DETAILED DESCRIPTION OF THE INVENTION

45 In accordance with the subject invention, plastid expression constructs are provided
30 which generally comprise a promoter functional in a plant plastid, a ribosome binding site derived from the T7 Bacteriophage Polymerase gene 10 leader, a DNA sequence encoding
50 for a gene of interest, and a transcription termination region capable of terminating

5 transcription in a plant plastid. These elements are provided as operably joined
components in the 5' to 3' direction of transcription.

10 Furthermore, the constructs of the present invention may also include a nucleic
acid sequence encoding a peptide capable of targeting said DNA sequence encoding a
5 protein to the thylakoid lumen within the chloroplast.

15 Of particular interest in the present invention are methods for the production of
proteins in a host plant cell plastid having a non-methionine N-terminus. Such methods
generally involve the use of fusion proteins having an N-terminus sequence which is
10 recognized by an endogenous protease. In particular, a DNA sequence encoding a
cleavable ubiquitin peptide is fused to a DNA sequence encoding a protein of interest.
After expression of the fusion protein in the plastid, an endogenous protease acts on the
20 fusion to cleave off the ubiquitin portion of the protein.

25 Also of interest in the present invention is the use of the plastid expression
constructs to direct the high level transcription and translation (expression) of nucleic acid
15 sequences. Such plastid expression constructs find use in directing the high level
expression of DNA sequences encoding for enzymes involved in herbicide tolerance or
encoding for the production of pharmaceutical proteins.

30 Of more particular interest in the present invention is the use of the plastid
expression constructs to direct the high level translation of transcribed messenger RNA.

35 20 DNA sequence and biochemical data reveal a similarity of the plastid organelle's
transcriptional and translational machineries and initiation signals to those found in
prokaryotic systems. In fact, plastid derived promoter sequences have been reported to
direct expression of reporter genes in prokaryotic cells. In addition, plastid genes are often
organized into polycistronic operons as they are in prokaryotes.

40 25 Despite the apparent similarities between plastids and prokaryotes, there exist
fundamental differences in the methods used to control gene expression in plastids and
prokaryotes. As opposed to the transcriptional control mechanisms typically observed in
prokaryotes, plastid gene expression is controlled predominantly at the level of translation
and mRNA stability by trans-acting nuclear encoded proteins.

45 30 Translation is a multi-stage process which first involves the binding of messenger
RNA (mRNA) to ribosomes. Beginning at the translation start codon, the mRNA codons
are read sequentially as the ribosomes move along the mRNA molecule. The specified
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5 amino acids are then sequentially added to the growing polypeptide chain to yield the protein or polypeptide encoded in the mRNA.

As mentioned, the first step in the translation process is the binding of the mRNA molecule to the ribosome. The nature of this interaction (i.e. binding) has been only
10 partially elucidated. Analysis of RNase-resistant oligonucleotides isolated from bacterial translation initiation complexes indicate that a RNA fragment approximately 30 to 40 nucleotides in length comprises the initial ribosome binding site (RBS). Thus, a RBS is
15 hereinafter understood to comprise a sequence of mRNA surrounding the translation start codon which is responsible for the binding of the ribosome and for initiation of translation.

10 Recently, ribosome binding sites have been identified which are capable of directing translation in a prokaryotes. For example, a ribosome binding site derived from
20 the T7 bacteriophage gene 10 leader, G10L (USPN 5,232,840, the entirety of which is incorporated herein by reference), has been identified which enhances expression of nucleic acid sequences in prokaryotes.

25 Herbicides such as N-phosphonomethylglycine, halogenated hydroxybenzonitriles, and norflurazon have been the subject of a large amount of investigation.

30 N-phosphonomethylglycine, commonly referred to as glyphosate, inhibits the shikimic acid pathway which leads to the biosynthesis of aromatic compounds including
35 amino acids, plant hormones and vitamins. Specifically, glyphosate curbs the conversion of phosphoenolpyruvic acid (PEP) and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (hereinafter referred to as EPSP synthase or EPSPS).

40 Glyphosate tolerant plants have been produced by transformation of various EPSP synthase genes into the nuclear genome of a plant. A gene for EPSP synthase has been
45 cloned from *Agrobacterium tumefaciens* sp strain CP4 (USPN 5,633,435) and confers a high level of glyphosate tolerance in plants. Furthermore, high levels of glyphosate tolerance has been achieved in a number of crop plants by fusing EPSPS to a chloroplast transit peptide (CTP) for targeted expression in plastids. In addition, variants of the wild-type EPSPS enzyme have been isolated which are glyphosate tolerant as a result of
50 alterations in the EPSPS amino acid coding sequence (Kishore and Shah, *Ann. Rev. Biochem.* (1988) 57:627-663; Shulze et al., *Arch. Microbiol.* (1984) 137:121-123; Kishore et al., *Fed. Proc.* (1986) 45:1506). These variants typically have a higher K_i for glyphosate than the wild-type EPSPS enzyme which confers the glyphosate tolerant phenotype, but

5 these variants are also characterized by a high K_m for PEP which makes the enzyme
kinetically less efficient (Kishore and Shah, *Ann. Rev. Biochem.* (1988) 57:627-663; Sost
et al., *FEBS Lett.* (1984) 173: 238-241; Shulze et al., *Arch. Microbiol.* (1984) 137:121-
123; Kishore et al., *Fed. Proc.* (1986) 45:1506; Sost and Amrhein, *Arch. Biochem.*
10 *Biophys.* (1990) 282: 433-436).

15 In addition to engineering plants for glyphosate tolerance, plants have also been
engineered to tolerate other classes of herbicides such as halogenated
hydroxybenzonitriles, and norflurazon using nucleic acid sequences expressed in the
nucleus.

20 10 Halogenated hydroxybenzonitriles, such as Bromoxynil, are suggested to act
herbically by inhibiting the quinone-binding protein complex of photosystem II,
inhibiting electron transfer (Van Rensen (1982) *Physiol. Plant* 54:515-520, and Sanders
and Pallett (1986) *Pestic. Biochem. Physiol.* 26:116-122). Herbicides such as norflurazon
inhibit the production of carotenoids.

25 15 Plants which are resistant to Bromoxynil have been produced by expressing DNA
sequences encoding for enzymes capable of detoxifying Bromoxynil (nitrilases) in the
plant cell nucleus. DNA sequences encoding for such nitrilases have been cloned from
bacteria such as *Klebsiella pneumoniae* and used to construct vectors to direct the
30 expression of the DNA sequence in plant cell nucleus (USPN 4,810,648, the entirety of
which is incorporated herein by reference).

35 20 Plants which are resistant to Norflurazon have been engineered by expressing
nucleic acid sequences which encode for enzymes in the carotenoid biosynthetic pathway
in plant cell nuclei. For example, expressing a phytoene desaturase from *Erwinia*
uredovora provides tolerance to norflurazon.

40 25 While plants transformed to express nucleic acid sequences encoding for such
enzymes from the nuclear genome have found utility in engineering herbicide tolerant
plants, it would be increasingly beneficial to obtain herbicide tolerant plants via plastidial
expression.

45 30 In the examples provided herein, DNA sequences encoding for enzymes involved
in herbicide tolerance are used in constructs to direct the expression of the sequences from
the plant plastid. DNA sequences encoding for 5-enolpyruvylshikimate-3-phosphate
synthase (EPSPS), bromoxynil nitrilase (Bxn), phytoene desaturase (crtI (Misawa et al,
50 (1993) *Plant Journal* 4:833-840, and (1994) *Plant Jour* 6:481-489), and acetohydroxyacid

5 synthase (AHAS (Sathasiivan *et al.* (1990) *Nucl. Acids Res.* 18:2188-2193)) are used in the expression constructs of the present invention to direct the expression of said herbicide tolerance nucleotide sequences from the plant plastid.

Transplastomic tobacco plants are identified which are homoplasmic for the DNA
10 sequences encoding the herbicide tolerance genes. Homoplasmic plants demonstrate a high level of protein expression from the plastid. Furthermore, homoplasmic plants demonstrate a high level of tolerance for the respective herbicide. For example, as described in more detail in the example below, plants transformed to express EPSPS from the plastid demonstrate a high level of tolerance for the herbicide glyphosate. In addition,
15 10 homoplasmic tobacco lines expressing nitrilase or phytoene desaturase demonstrate high levels of tolerance for the herbicides bromoxynil and norflurazon, respectively.

An artisan skilled in the art to which the present invention pertains will recognize that additional sequences may be employed to in the plastid expression constructs of the instant invention to produce herbicide tolerant plants. Other nucleic acid sequence which
20 25 may find use in the plastid expression constructs herbicide tolerant plants include the *bar* gene for tolerance to glufosinate (DeBlock, *et al.* (1987) *EMBO J.* 6:2513-2519).

Furthermore, additional glyphosate tolerance genes may be employed in the constructs of the present invention. Additional glyphosate tolerant EPSPS genes are described in U.S. Patent Number 5,627,061, Padgett *et al.* (1996) *Herbicide Resistant Crops*, Lewis Publishers, 53-85, and in Penaloza-Vazquez, *et al.* (1995) *Plant Cell Reports* 14:482-487, the entireties of which are incorporated herein by reference.

It should be noted that the herbicide tolerance constructs of the present invention may also include sequences encoding genes involved in other stress tolerance genes, for example insect or disease resistance/tolerance genes. As described in more detail in the examples that follow, plastid expression constructs are used to regenerate plants which are resistant to the herbicide Buctril, and which also express the *Bacillus thuringensis* *cry1Ac* protein.
35 40

In addition, the plastid expression constructs also find use in directing the production of human biological proteins (pharmaceutical proteins) from the plant plastid.
45 30 As set forth in detail in the examples, constructs are provided for expression of aprotinin and human growth hormone in the plant plastid. Other sequences which may find use in the expression constructs of the present invention for the production of human biologics include sequences encoding for insulin or insulin precursors. However, the skilled artisan
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5 will recognize that many nucleotide sequences encoding for human biologics may be
employed in the constructs of the present invention to direct their expression from a plant
plastid such as those described in Goodman and Gelman (1990) *Pharmacological Basis of*
Therapeutics, Pergamon Press, 8th Edition, Sections 14 and 15. As, it is contemplated that
10 5 any protein for which the nucleotide sequence has been identified can be used in the
constructs of the present invention.

The present invention also provides methods for producing a pharmaceutical
15 protein with a non-methionine N-terminus in a plant plastid. In general, the methods
comprise expressing a fusion protein including a ubiquitin gene fused to a protein of
20 interest in a plastid. The ubiquitin gene is obtained from a natural source and cloned into
an appropriate vector, as described in WO 88/02406, *supra*, the disclosure of which is
25 incorporated herein by reference, or it is synthesized chemically, using, e.g., the method
described by Ecker et al., *J. Biol. Chem.*, 262:3524-3527 (1987) and Ecker et al., *J. Biol.*
Chem., 262: 14213-14221 (1987), the disclosures of which are incorporated by reference.
30 15 The ubiquitin fusion proteins are recognized by ubiquitin protease, contrary to previous
reports (Vierstra (1996) *Plant Mol. Biol.* 32:275-302), which cleaves immediately
downstream of the carboxy terminal glycine residue of ubiquitin. This property has
allowed production of recombinant proteins containing N-terminal residues other than
methionine (Baker (1996) *Current Opin. Biotech.* 7:541-546).

20 20 Additional methods for the production of pharmaceutical proteins with a non-
methionine N-terminus in a plant plastid are also provided. As described more fully in the
35 Examples below, constructs are prepared to direct the production of a methionine-hGH
(M-hGH) in a plant cell plastid. The constructs generally comprise a transcriptional
40 initiation region and a DNA sequence encoding hGH. Surprisingly, N-terminal amino
acid sequencing of the extracted hGH produced in transplastomic plants reveals that the N-
45 terminal methionine is cleaved from the mature hGH protein, producing hGH with an
alanine N-terminus (A-hGH). This result indicates the interaction of the expressed hGH
with a methionine amino peptidase (MAP) in the plant cell. While it is anticipated that
any amino acid may follow in the N-terminal methionine, the second amino acid is
50 30 preferably selected from the group consisting of alanine, cysteine, glycine, proline, serine,
threonine, and valine.

As described in more detail below, nucleic acid sequences encoding for the human
55 growth hormone (hGH) are employed in plastid expression constructs of the present

5 invention. Further, transplastomic tobacco plants containing such constructs demonstrate a high level of expression of hGH. In addition, the hGH protein expressed from the plant plastid exhibits characteristics of proper processing as well as proper protein folding.

Human growth hormone (hGH) participates in much of the regulation of normal
10 human growth and development. This 22,000 dalton pituitary hormone exhibits a multitude of biological effects including linear growth (somatogenesis), lactation, activation of macrophages, insulin-like and diabetogenic effects among others (Chawla,
15 *Ann. Rev. Med.* (1983) 34:519; Edwards, *et al.*, *Science* (1988) 239:769; Thorner *et al.*, *J. Clin. Invest.* (1988) 81:745). hGH is a member of a family of homologous hormones that
20 include placental lactogens, prolactins, and other genetic and species variants or growth hormone (Nicoll, *et al.*, *Endocrine Reviews* (1986) 7:169). hGH is unusual among these in that it exhibits broad species specificity and binds to either the cloned somatogenic
25 (Leung, *et al.*, *Nature* (1987) 33:537) or prolactin receptor (Boutin, *et al.*, *Cell* (1988)
53:69). The primary use of hGH is in the treatment of hypopituitary dwarfism in children.
30 Additional indications are in treatment of Turner syndrome, chronic renal failure, HIV
wasting syndrome and the treatment of the elderly and critically ill (Tritos, *et al.* (1998)
Am. J. Med. 105:44-57).

As produced in the pituitary gland, hGH enters the secretory system, coincident
35 with removal of its signal peptide and formation of two disulfide bonds (Chawla, *et al.*
20 (1983) *supra*). In the pituitary gland, removal of the signal peptide from hGH (also referred to as human somatotropin or hST) during secretion leaves phenylalanine as the N-terminal amino acid (Chawla, *et al.* (1983) *Annu. Rev. Med.* 34:519-547). As normal translation in plastids initiates at methionine, a ubiquitin-hGH fusion was designed to yield a phenylalanine N-terminus (F-hGH) in the final hGH product.

40 Surprisingly, although ubiquitin protease was previously reported to not be present in chloroplasts (Vierstra (1996) *Plant Mol. Biol.* 32:275-302), the ubiquitin-hGH fusion was processed during synthesis, accumulation or purification from the plants to produce a phenylalanine N-terminus hGH product (F-hGH). The control construct carrying the full-length cDNA encoded methionine and alanine as the first amino acids of hGH.

45 30 As described in the Examples below, constructs comprising nucleic acid sequences encoding aprotinin (also known as bovine pancreatic trypsin inhibitor, BPTI) were employed in plastid expression constructs of the present invention. Aprotinin is a basic protein present in several bovine organs and tissues, such as the lymph nodes, pancreas,

5 lungs, parotid gland, spleen and liver. Aprotinin is known to inhibit various serine
proteases, including trypsin, chymotrypsin, plasmin and kallikrein, and is used
therapeutically in the treatment of acute pancreatitis, various stages of shock syndrome,
hyperfibrinolytic hemorrhage and myocardial infarction. In addition, administration of
10 5 aprotinin in high doses significantly reduces blood loss in connection with cardiac surgery,
including cardiopulmonary bypass (Bidstrup, *et al.* (1989) *Cardiovasc Surg.* 44:640-645)

15 In developing the constructs, the various fragments comprising the regulatory
regions and open reading frame may be subjected to different processing conditions, such
as ligation, restriction enzyme digestion, PCR, *in vitro* mutagenesis, linkers and adapters
20 10 addition, and the like. Thus, nucleotide transitions, transversions, insertions, deletions, or
the like, may be performed on the DNA which is employed in the regulatory regions or the
DNA sequences of interest for expression in the plastids. Methods for restriction digests,
Klenow blunt end treatments, ligations, and the like are well known to those in the art and
25 20 are described, for example, by Maniatis *et al.* (*in Molecular Cloning: A Laboratory
Manual* (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

25 During the preparation of the constructs, the various fragments of DNA will often
be cloned in an appropriate cloning vector, which allows for amplification of the DNA,
modification of the DNA or manipulation of the DNA by joining or removing sequences,
30 30 linkers, or the like. Preferably, the vectors will be capable of replication to at least a
relatively high copy number in *E. coli*. A number of vectors are readily available for
cloning, including such vectors as pBR322, vectors of the pUC series, the M13 series
35 35 vectors, and pBluescript vectors (Stratagene; La Jolla, CA).

40 In order to provide a means of selecting the desired plant cells, vectors for plastid
transformation typically contain a construct which provides for expression of a selectable
45 25 marker gene. Marker genes are plant-expressible DNA sequences which express a
polypeptide which resists a natural inhibition by, attenuates, or inactivates a selective
substance, including, but not limited to, antibiotic, herbicide etc.

45 Alternatively, a marker gene may provide some other visibly reactive response, i.e.,
may cause a distinctive appearance or growth pattern relative to plants or plant cells not
30 30 expressing the selectable marker gene in the presence of some substance, either as applied
directly to the plant or plant cells or as present in the plant or plant cell growth media.

50 In either case, the plants or plant cells containing such selectable marker genes will
have a distinctive phenotype for purposes of identification, i.e., they will be

5 distinguishable from non-transformed cells. The characteristic phenotype allows the identification of cells, cell groups, tissues, organs, plant parts or whole plants containing the construct.

10 Detection of the marker phenotype makes possible the selection of cells having a 5 second gene to which the marker gene has been linked. This second gene typically comprises a desirable phenotype which is not readily identifiable in transformed cells, but which is present when the plant cell or derivative thereof is grown to maturity, even under 15 conditions wherein the selectable marker phenotype itself is not apparent.

The use of such a marker for identification of plant cells containing a plastid 20 construct has been described by Svab *et al.* (1993, *supra*). In the examples provided below, a bacterial *aadA* gene is expressed as the marker under the regulatory control of chloroplast 5' promoter and 3' transcription termination regions, specifically the regulatory 25 regions of the *psbA* gene (described in Staub *et al.*, *EMBO J.*(1993) 12(2):601-606). Numerous additional promoter regions can also be used to drive expression of the 30 selectable marker gene, including various plastid promoters and bacterial promoters which have been shown to function in plant plastids.

35 Expression of the *aadA* gene confers resistance to spectinomycin and streptomycin, and thus allows for the identification of plant cells expressing this marker. The *aadA* gene product allows for continued growth and greening of cells whose chloroplasts comprise the 40 selectable marker gene product. Cells which do not contain the selectable marker gene product are bleached. Selection for the *aadA* gene marker is thus based on identification 45 of plant cells which are not bleached by the presence of streptomycin, or more preferably spectinomycin, in the plant growth medium.

50 A number of markers have been developed for use with plant cells, such as 45 resistance to chloramphenicol, the aminoglycoside G418, hygromycin, or the like. Other genes which encode a product involved in chloroplast metabolism may also be used as 55 selectable markers. For example, genes which provide resistance to plant herbicides such as glyphosate, bromoxynil or imidazolinone may find particular use. Such genes have been reported (Stalker *et al.*, *J. Biol. Chem.* (1985) 260:4724-4728 (glyphosate resistant EPSP); Stalker *et al.*, *J. Biol. Chem.* (1985) 263:6310-6314 (bromoxynil resistant nitrilase gene); and Sathasivan *et al.*, *Nucl. Acids Res.* (1990) 18:2188 (AHAS imidazolinone resistance gene)).

5 Stable transformation of tobacco plastid genomes by particle bombardment is
reported (Svab *et. al.* (1990), *supra*) and Svab *et al.* (1993), *supra*). The methods
described therein may be employed to obtain plants homoplasmic for plastid expression
constructs.

10 5 Generally, bombarded tissue is cultured for approximately 2 days on a cell
division-promoting media, after which the plant tissue is transferred to a selective media
containing an inhibitory amount of the particular selective agent, as well as the particular
15 10 hormones and other substances necessary to obtain regeneration for that particular plant
species. Shoots are then subcultured on the same selective media to ensure production and
selection of homoplastic shoots.

20 20 Transplastomic tobacco plants are analyzed for a pure population of transformed
plastid genomes (homoplastic lines). Homoplasmy is verified using Southern analysis
employing nucleic acid probes spanning a region of the transgene and chloroplast genome
(i.e. the insertion region). Transplastomic plants which are heteroplastic (i.e. contain a
25 25 mixture of plastid genomes containing and lacking the transgene) are characterized by a
hybridization pattern of wild type and transgenic bands. Homoplastic plants show a
hybridization pattern lacking the wild type band.

30 30 Alternatively, homoplasmy may be verified using the polymerase chain reaction
(PCR). PCR primers are utilized which are targeted to amplify from sequences from the
20 35 insertion region. For example, a pair of primers may be utilized in a PCR reaction. One
primer amplifies from a region in the transgene, while the second primer amplifies from a
region proximal to the insertion region towards the insertion region. A second PCR
reaction is performed using primers designed to amplify the region of insertion.
Transplastomic lines identified as homoplastic produce the expected size fragment in the
25 40 first reaction, while they do not produce the predicted size fragment in the second reaction.

40 45 Where transformation and regeneration methods have been adapted for a given
plant species, either by *Agrobacterium*-mediated transformation, bombardment or some
other method, the established techniques may be modified for use in selection and
regeneration methods to produce plastid-transformed plants. For example, the methods
45 50 described herein for tobacco are readily adaptable to other solanaceous species, such as
tomato, petunia and potato.

50 For transformation of soybean, particle bombardment as well as *Agrobacterium*-
mediated nuclear transformation and regeneration protocols have been described (Hinchee

5 *et al.* USPN 5,416,011, and Christou *et al.* USPN 5,015,580). The skilled artisan will
recognize that protocols described for soybean transformation may be used

10 In *Brassica*, *Agrobacterium*-mediated transformation and regeneration protocols
generally involve the use of hypocotyl tissue, a non-green tissue which might contain a low
15 5 plastid content. Thus, for *Brassica*, preferred target tissues would include microspore-
derived hypocotyl or cotyledonary tissues (which are green and thus contain numerous
plastids) or leaf tissue explants. While the regeneration rates from such tissues may be
low, positional effects, such as seen with *Agrobacterium*-mediated transformation, are not
15 10 expected, thus it would not be necessary to screen numerous successfully transformed
plants in order to obtain a desired phenotype.

20 For cotton, transformation of *Gossypium hirsutum* L. cotyledons by co-cultivation
with *Agrobacterium tumefaciens* has been described by Firoozabady *et al.*, *Plant Mol. Bio.*
(1987) 10: 105-116 and Umbeck *et al.*, *Bio/Technology* (1987) 5:263-266. Again, as for
Brassica, this tissue may contain insufficient plastid content for chloroplast
25 15 transformation. Thus, as for *Brassica*, an alternative method for transformation and
regeneration of alternative target tissue containing chloroplasts may be desirable, for
instance targeting green embryogenic tissue.

30 Other plant species may be similarly transformed using related techniques.
Alternatively, microprojectile bombardment methods, such as described by Klein *et al.*
20 20 (*Bio/Technology* 10:286-291) may also be used to obtain nuclear transformed plants
comprising the viral single subunit RNA polymerase expression constructs described
35 35 herein. Cotton transformation by particle bombardment is reported in WO 92/15675,
published September 17, 1992. Suitable plants for the practice of the present invention
include, but are not limited to, soybean, cotton, alfalfa, oil seed rape, flax, tomato, sugar
25 40 beet, sunflower, potato, tobacco, *maize*, wheat, rice and lettuce.

40 The vectors for use in plastid transformation preferably include means for
providing a stable transfer of the plastid expression construct and selectable marker
construct into the plastid genome. This is most conveniently provided by regions of
45 45 homology to the target plastid genome. The regions of homology flank the construct to be
transferred and provide for transfer to the plastid genome by homologous recombination,
via a double crossover into the genome. The complete DNA sequence of the plastid
50 50 genome of tobacco has been reported (Shinozaki *et al.*, *EMBO J.* (1986) 5:2043-2049).
Complete DNA sequences of the plastid genomes from liverwort (Ohya *et al.*, *Nature*

5 (1986) 322:572-574) and rice (Hiratsuka *et al.*, *Mol. Genet.* (1989) 217:185-194),
have also been reported.

10 Where the regions of homology are present in the inverted repeat regions of the
plastid genome (known as IRA and IRB), two copies of the transgene are expected per
5 transformed plastid. Where the regions of homology are present outside the inverted
repeat regions of the plastid genome, one copy of the transgene is expected per
transformed plastid. The regions of homology within the plastid genome are
15 approximately 1kb in size. Smaller regions of homology may also be used, and as little as
100 bp can provide for homologous recombination into the plastid genome. However, the
frequency of recombination and thus the frequency of obtaining plants having transformed
10 plastids decreases with decreasing size of the homology regions.

20 Examples of constructs having regions of homology within the plastid genome are
described in Svab *et.al.* (1990 *supra*), Svab *et al.* (1993 *supra*) and Zoubenko *et al.* (*Nuc
Acid Res* (1994) 22(19):3819-3824).

25 15 As described in more detail in the examples below, constructs are described which
provide for enhanced expression of DNA sequences in plant plastids. Various
30 promoter/ribosome binding site sequences are employed to direct expression in plant
plastids. Promoter sequences of the 16S ribosomal RNA operon (*Prrn*) are linked to a
ribosome binding site (RBS) derived from the T7 bacteriophage gene 10 leader sequence
35 (G10L). DNA sequences expressed under the regulatory control of the *Prrn*/G10L
sequence show a significantly higher level of protein expression than those levels obtained
under the control of other promoter/RBS combinations, while expression of mRNA may or
may not be higher in these plants.

40 In the examples below, nucleic acid sequences encoding CP4 EPSP synthase
25 (USPN 5,633,435) are placed into expression constructs for expression of EPSP synthase
enzyme from the plant plastid. Furthermore, a DNA sequence encoding for hGH (USPN
45 5,424,199) is also placed into expression construct for the expression of human growth
hormone from the plant plastid. The constructs prepared utilize a ribosome binding site
designed after the T7 bacteriophage gene 10 leader (G10L) to increase the expression of
30 the nucleic acid sequences in the plant plastid.

Plastid expression constructs encoding for the expression of EPSPS and hGH are
introduced via a chloroplast transformation vector.

50

5 Tobacco lines containing the native encoding sequence to the EPSPS enzyme
expressed in plastids under the control of the *Prrn/G10L* promoter/ribosome binding site
sequence demonstrate a significantly higher level of protein expression than those levels
obtained from EPSPS expressed under the control of the *Prrn/rbcL* RBS sequence.

10 5 However, EPSPS mRNA is expressed at a higher level in plants expressing CP4 EPSPS
from the plastid under the control of the *Prrn/rbcL*(RBS). These results indicate that
translation from transcripts containing the T7 bacteriophage gene 10 ribosome binding site
is more efficient. In addition, protein expression levels of EPSPS obtained from
15 transplastomic tobacco lines expressing EPSPS under the control of the *Prrn/G10L* RBS
provide for a high level of glyphosate tolerance.

20 Furthermore, transplastomic tobacco lines transformed to express hGH under the
control of the *Prrn/G10L* promoter/ribosome binding site sequence demonstrate a
significantly higher level of protein expression than those levels obtained from hGH
expressed under the control of the *PpsbA* promoter/RBS sequence.

25 15 Increases in protein expression levels of at least approximately 200 fold may be
obtained from constructs utilizing *Prrn/G10L* ribosome binding site for expression of
EPSPS and hGH over the expression levels obtained from other promoter/RBS
combinations for plastid expression. In addition, protein levels obtained from plastid
30 expression constructs utilizing the *Prrn/G10L* promoter/RBS sequence may accumulate 50
to 3500 fold higher levels than from nuclear expression constructs. Thus, inclusion of the
20 G10L ribosome binding site in plastid expression constructs may find use for increasing
the levels of protein expression from plant plastids.

35 Furthermore, the constructs of the present invention may also include sequences to
target the expressed protein to a particular suborganellar region, for example, the thylakoid
40 45 lumen of the chloroplast. For example, as described in the examples below, a nucleotide
sequence encoding a peptide from the plastid genome cytochrome f targets the expressed
aprotinin protein to the thylakoid membrane. Such targeting of expressed proteins may
provide for a compartmentalization of the protein allowing for increased oxidative stability
and proper protein folding.

40 30 The invention now being generally described, it will be more readily understood by
reference to the following examples which are included for purposes of illustration only
50 and are not intended to limit the present invention.

5

EXAMPLES**Example 1 Expression Constructs**

10 Constructs and methods for use in transforming the plastids of higher plants are described in Zoubenko *et al.* (*Nuc Acid Res* (1994) 22(19):3819-3824), Svab *et al.* (*Proc. Natl. Acad. Sci.* (1990) 87:8526-8530 and *Proc. Natl. Acad. Sci.* (1993) 90:913-917) and Staub *et al.* (*EMBO J.* (1993) 12:601-606). Constructs and methods for use in transforming plastids of higher plants to express DNA sequences under the control of a

15 nuclearly encoded, plastid targeted T7 polymerase are described in U.S. Patent Number 5,576,198. The complete DNA sequences of the plastid genome of tobacco are reported by Shinozaki *et al.* (*EMBO J.* (1986) 5:2043-2049). All plastid DNA references in the following description are to the nucleotide number from tobacco.

20 The complete nucleotide sequence encoding the tobacco cytochrome *f* (*petA*) is described in Bassham *et al.*, (1991) *J Biol Chem* 266:23606-23610 and Konishi *et al.* (1993) *Plant Cell Physiol* 34:1081-1087.

1A. Promoter/Ribosome Binding Site Sequences

30 The promoter region of the plastid 16S ribosomal RNA operon (*Prrn*) is linked to a synthetic ribosome binding site (RBS) patterned on the plastid *rbcL* gene leader to create the *Prrn/rbcLRBS* fragment. The *Prrn/rbcLRBS* sequence is as described in Svab *et al.* (1993, *supra*) for the *Prrn/rbcL(S)* fragment.

35 The promoter region of the plastid *psbA* promoter (*PpsbA*) and terminator sequences (*TpsbA*) are described in Staub *et al.* (1993, *EMBO J.*, 12, 601-606).

40 The *Prrn/G10L* sequence was constructed by annealing two oligonucleotide sequences, T7lead1 and T7lead2 (Table 1), to create the G10L plastid ribosome binding site (Figure 1). The G10L sequence was ligated to the 3' terminus of the *Prrn* promoter sequence as an *EcoRI/NcoI* fragment to create the *Prrn/G10L* sequence.

45

50

5

Table 1

10

	T7lead1	5'-AAT TGT AGA AAT AAT TTT GTT TAA CTT TAA GAA GGA
		GAT ATA CC-3'
5	T7lead2	5'-CAT GGG TAT ATC TCC TTC TTA AAG TTA AAC AAA ATT
		ATT TCT AC-3'

15

Chimeric genes are preferably inserted into the expression vector to direct their transcription from the *Prrn* promoter. Thus, in the plastid genome, chimeric genes are transcribed from the *Prrn/RBS* promoter, or the *Prrn/G10L* promoter in the plant plastid.

20

IB. CP4 EPSPS Plastid Expression Constructs

25

A plastid expression vector pMON30117 is constructed from a precursor vector pPRV111B (Zoubenko, *et al.* 1994, *supra*, GenBank accession U12813). The vector pMON30117 carries a multiple cloning site for insertion of a passenger gene in a *Prrn/rbcLRBS/T_{rps16}* expression cassette. The *Prrn/rbcLRBS* sequence is cloned into pPRV111B vector as an *EcoRI/NcoI* fragment, and the terminator region from the plastid *rps16* gene (*T_{rps16}*) is cloned 3' of the *Prrn* promoter as a *HindIII/NcoI* fragment. The *T_{rps16}* fragment comprises the *rps16* gene 3'-regulatory region from nucleotides 5,087 to 4,939 in the tobacco plasmid DNA.

30

20 The pPRV111B backbone of the vector pMON30117 contains a marker gene, *aadA*, for selection on spectinomycin and streptomycin, and *rps 7/12* for the integration, by homologous recombination, of the passenger DNA into *rrnV-rps7/12* intergenic region.

35

The plastid expression construct pMON30118 was prepared by cloning the native CP4 EPSPS gene fused with the N-terminal five (5) amino acids from the plastid *rbcL* (described in Svab *et al.*, 1993 *supra*) gene as an *NcoI/SmaI* fragment into the multiple cloning site of the vector pMON30117.

40

45 The plastid expression construct pMON30123 is essentially the same as pMON30118 with the exception of the deletion of the N-terminal five (5) amino acids from the plastid *rbcL*.

50

30 The plastid expression construct pMON30130 was created by replacing the native CP4 EPSPS of pMON30123, with a synthetic CP4 gene. This construct also lacks the N-terminal 5 amino acid fusion from the plastid *rbcL* gene.

- 5 The plastid expression construct pMON38773 was constructed by replacing the
Prrn/RBS sequence of pMON30123 with the Prrn/G10L promoter sequence described
above. The EPSPS DNA sequence of pMON38773 also lacks the N-terminal 5 amino acid
fusion from the plastid rbcL gene.
- 10 5 A plastid expression construct, pMON38766 was constructed using the promoter
from T7 phage gene 10 (P-T7), including G10L, CP4 (native) gene coding region, and the
terminator sequence from plastid rps16 gene (Trps16).
- 15 10 A plastid expression construct, pMON38797 was constructed using the promoter
from T7 phage gene 10 (P-T7), including G10L, CP4 (synthetic) gene coding region,
10 terminator from plastid rps16 gene (Trps16).
- 20 15 A plastid expression construct, pMON38798 was constructed using the promoter
of the 16SrDNA operon (Prrn), G10L, CP4 (synthetic) gene coding region, terminator
from plastid rps16 gene (Trps16).
- 25 20 A plastid expression construct, pMON38793 was constructed using the promoter
15 of the 16SrDNA operon (Prrn), a synthetic ribosome binding site (RBS) patterned from the
plastid rbcL gene, the glyphosate tolerant Petunia EPSP synthase gene (P-EPSPS,
Padgett, et al. (1987) *Arch. Biochem. Biophys.* 258:564-573) carrying the mutation
Glycine to Alanine at amino acid position 101, terminator from plastid rps16 gene
30 (Trps16).
- 35 20 A plastid expression construct, pMON38796 was constructed using the promoter
of the 16SrDNA operon (Prrn), synthetic ribosome binding site (RBS) patterned from the
plastid rbcL gene, the glyphosate tolerant Achromobacter (strain LBAA) EPSP synthase
gene (U.S. Patent Number 5,627,061, the entirety of which is incorporated herein by
reference) carrying the mutation Glycine to Alanine at amino acid position 100, terminator
40 25 from plastid rps16 gene (Trps16).
- 45 30 A plastid expression construct, pMON45204, was constructed using the promoter
of the 16SrDNA operon (Prrn) with the G10L, the glyphosate tolerant Pseudomonas (strain
LBAA) EPSP synthase gene carrying the mutation Glycine to Alanine at amino acid
position 100, terminator from plastid rps16 gene (Trps16).
- 50 35 A plastid expression construct, pMON45201, was constructed using the promoter
of the 16SrDNA operon (Prrn), synthetic ribosome binding site (RBS) patterned from the
plastid rbcL gene, wild-type glyphosate tolerant *Bacillus subtilis* aroE (EPSPS) (U.S.
Patent Number 5,627,061) gene, terminator from plastid rps16 gene (Trps16).

5

1C. Bucril (bxn) Plastid Expression Constructs

The *bxn* herbicide resistance gene (U.S. Patent Number 4,810,648, the entirety of which is incorporated herein by reference) was removed from the plasmid pBrx47 as an 10 restriction fragment and cloned into *Nco*/*Asp*718 cut pUC120 resulting in 15 plasmid pBrx87. Plasmid pBrx87 was then digested with *Nco*/*Xba* and cloned into the *Nco*/*Xba* sites of the plasmid pLAA21 which contains the *Prrm* plastid promoter and the *rpsL* 3' region for plastid expression. The resulting plasmid was designated pBrx89. 20 Plasmid pBrx89 was digested with *Sac* I and *Hind* III and the 1.5 kb chimeric *bxn* gene 25 with plastid expression signals was inserted into the *Sac/Hind* III sites of the tobacco plastid homology vector pOVZ44B (Zoubenko et al, Nuc Acids Res 22: 3819-3824 30 (1994)) to create plasmid pCGN5175.

To construct plasmid pCGN6114, plasmid pBrx90 (a Bluescript plasmid containing the *bxn* gene encoding the bromoxynil specific nitrilase) was digested with *Nco* 35 *I/Asc* I and the *bxn* structural gene was substituted for the GUS gene in the *Nco/Asc* I digested plasmid pCGN5063 resulting in plasmid pCGN6107. This plasmid contains the *bxn* gene under the control of the T7 promoter/gene10 leader at the 5' end and the *psbA/T7* hybrid transcriptional terminator at the 3' end of the chimeric gene. This T7 40 promoter/*bxn* chimeric gene was excised from pCGN6107 as a *Hind* III/*Not* I DNA segment and moved into the chloromphenical plasmid BCSK+ (Stratagene) at the *Hind* III/*Not* sites to create plasmid pCGN6109. The chimeric gene was then moved as a *Hind* III/*Not* fragment from pCGN6109 into the chloroplast homology vector pOVZ44B 45 described above to create plasmid pCGN6114. Tobacco plants transformed with pCGN6114 require the T7 RNA polymerase be provided in the plant plastid background to activate transcription of the chimeric *bxn* gene via the T7 promoter. This system has previously been detailed in McBride et al., PNAS, 91:7301-7305 (1994) and McBride et 50 al., US Patent Number 5,576,198.

45

1D. BXN/AHAS Plastid Expression Constructs

A plastid expression construct, pCGN5026, is prepared to direct the expression of BXN and AHAS from the plant plastid. The AHAS nucleotide sequence (described in EP 55 Publication Number 0 525 384 A2, the entirety of which is incorporated herein by reference) is translationally linked to the BXN nucleotide sequence (U.S. Patent Number

4,810,648, the entirety of which is incorporated herein by reference). The AHAS structural
5 gene encoding acetohydroxyacid synthase was cloned from the plasmid pCGN4277 as an
10 *Nco* I to *Age* DNA fragment into the *Nco/Xma* sites of plasmid pUC120 to create plasmid
15 pCGN5022. This plasmid was then digested with the enzymes *Bam*H I and *Pst* and a 1.3
20 kb *Bam/Pst* DNA segment containing the *bxn* gene encoding the bromoxynil-specific
nitrilase was excised from the plasmid pBrx26 and cloned into the *Bam/Pst* sites of
25 pCGN5022 to create plasmid pCGN5023. Plasmid pCGN5023 contained a 3.3 kb DNA
segment containing the AHAS/*bxn* operon segment and this fragment. This plasmid was
30 cut at the unique *Pst* site and this *Pst* site was removed and replaced with a synthetic linker
containing a unique *Xba* I restriction site generating plasmid pCGN5024. Plasmid
35 pCGN5024 was digested with *Nco/Xba* and the 3.3 kb *Nco/Xba* DNA fragment was
cloned into the plastid promoter cassette vector pLAA21(*Pst*) that had been digested with
40 *Nco* and *Xba* to remove the GUS gene. The plasmid resulting from this cloning was
designated plasmid pCGN5025 and contained the herbicide operon under the control of
45 the plastid promoter *Prrn* and the *rpsL* 3' DNA segment. The entire chimeric herbicide
operon under the control of the plastid expression elements was excised from pCGN5025
as a *Sac* I /*Pst* DNA fragment and cloned into the *Sac/Pst* sites of the plastid homology
50 cassette vector pOVZ44B (Zoubenko *et al.*, *Nuc Acids Res* 22:3819-3824 (1994)) to
facilitate transfer into the tobacco chloroplast genome.

20

IE. Bt cryIAc and bxn Plastid Expression Construct

35 Plasmid pBrx9 (Stalker and McBride, (1987) *J Bacteriol* 169:955-960), an original
clone from *Klebsiella* containing a *bxn* gene DNA segment, was used as a template to
40 generate an ~450 bp *Bam*H I/*Cla* I PCR DNA fragment that encompasses the N-terminal
end of the *bxn* gene and includes 44 bp of the 5' untranslated portion of the native gene.
45 This fragment was exchanged with the ~400 bp *Bam/Cla* fragment in the plasmid pBrx90
resulting in plasmid pBrx90.1. This plasmid contains the entire *bxn* gene and the 44 bp
50 untranslated 5' DNA segment. The *bxn* gene was excised from plasmid pBrx90.1 as a
Bam/Asc I DNA segment and inserted into plasmid pCGN5146 at the *Bgl* II/*Asc* I sites to
generate plasmid pCGN5191. Plasmid pCGN5146 is a pKK233-2 (Pharmacia) derivative
containing the full-length *cryIAc* gene encoding the HD-73 Bt protoxin. Plasmid
55 pCGN5191 then contains the *cryIAc* and *bxn* genes in an operon configuration with the
bxn gene being the distal gene in the operon. Both genes are under the control of the *Piac*

5 promoter for *E coli* expression in 5191. Plasmid pCGN5191 was digested with *Nco*/*Asc* and the *Nco*/*Asc* DNA fragment containing the *Bt/bxn* operon was cloned into the *Nco*/*Asc* sites of the chloroplast homology vector pCGN5155, a derivative of pOVZ44B. The resulting plasmid, pCGN5197 contains the *Bt/bxn* operon under the control of the *Prrn*
10 5 plastid promoter and *rpsL* transcription terminator regions. This plasmid facilitated transfer of the *Bt/bxn* chimeric operon into the tobacco plastid genome.

15 ***1F. Phytoene desaturase Plastid Expression Constructs***

The *crtI* gene was obtained as a *Hind* III/*Sal* I PCR fragment from the original
10 10 plasmid containing the *Erwinia carotovora crt* operon (Misawa et al, (1994) *Plant Jour*
6:481-489)) and cloned as a *Hind* III/*Sal* DNA segment into BCSK+ (Stratagene) at the
20 20 *Hind* III/*Sal* sites to generate plasmid pCGN5172. The *crtI* fragment was cloned from
pCGN5172 as an *Nco* I/*Sal* I fragment into pCGN5038 (a derivative of pOVZ44B) to
create the plastid expression construct pCGN5177. This construct directs the expression
25 15 of the *crtI* sequence from the *Prrn* promoter and the *rps16* terminator sequence. This
plasmid facilitated the transfer of the chimeric *crtI* gene into the tobacco plastid genome.

30 ***1G. hGH Expression Constructs for Plant Transformation***

Nuclear Expression Constructs

20 20 The construct pWRG4747 was constructed to direct the expression of hGH in the
plant nuclear genome. This vector contains the hGH operably linked to the Figwort
35 35 Mosaic Virus promoter (USPN 5,378,619, the entirety is incorporated herein by reference)
and the CTP2 leader for directing the hGH protein into the plastid. The
MFV/CTP2L::hGH::NpA fragment is cloned along with the DNA sequence conferring
40 25 resistance to Kanamycin between the right and left borders (RB and LB) of the transfer
DNA (tDNA) of *Agrobacterium tumefaciens* to direct the integration into the nuclear
genome.

45 45 The nuclear transformation vector pWRG4744 contains essentially the same
elements as pWRG4747 except the construct lacks the CTP2 leader and the hGH protein is
30 30 directed to the plant cell cytoplasm.

50

5 Plastid Expression Constructs

The plastid expression vector pWRG4838 was constructed using the full length hGH gene expressed from the promoter and terminator region from the psbA gene, PpsbA and TpsbA, respectively (described in Staub *et al.* (1993), *supra*). This chimeric
10 5 promoter-gene-terminator fusion (PpsbA::hGH: :TpsbA) is cloned adjacent to the selectable marker gene *aadA* also driven by the plastid expression elements of the psbA gene. The two chimeric gene sequences are cloned into a vector between two sequences which direct the integration of the chimeric gene sequences into the tobacco plastid genome upstream of the plastid 16SrDNA. This is joined to a 1 kb Ampicillin resistance
15 10 gene which provides for selection of *E. coli* containing the construct and the pUC origin of replication for plasmid maintenance in *E. coli*.

20 The plastid expression construct pMON38755 was prepared using the hGH DNA sequence translationally fused at the N-terminus with the yeast ubiquitin gene (Ozkaynak, *et al.* (1984) *Nature* 312:663-666), creating the Ubi-hGH fusion gene. The Ubi-hGH
25 15 fusion gene is cloned next to the *aadA* gene for selection of transplastomic tobacco on media containing spectinomycin or streptomycin (from pPRV112B described in Zoubenko *et al.* (1994) *supra*). Sequences are included for the homologous recombination of sequences encoding for hGH and *aadA* expression. These sequences are obtained from the vector pPRV112B described in Zoubenko *et al.* (1994, *supra*). These sequences are
30 20 joined to a 1 kb ampicillin resistance gene which provides for selection of *E. coli* containing the construct and the pUC origin of replication for plasmid maintenance in *E. coli*.
35

40 The plastid expression construct pMON38794 contains essentially the same elements as pMON38755, with the exception that the 0.15 kb psbA promoter sequence is replaced with the *Prrn/G10L* promoter sequence described above.

1H. Constructs for the Expression of Aprotinin in Plastids

A series of constructs were prepared to direct the expression of the pharmaceutical protein aprotinin from the plastid. The nucleic acid sequence encoding for aprotinin
45 45 (Figure 2) was cloned into a plastid expression construct to control the expression of aprotinin from the T7 gene 10 leader promoter which is induced from a nuclearly
50 30 expressed, plastid targeted T7 Polymerase. The constructs used in which the aprotinin sequence was cloned are as described in U.S. Patent Number 5,576,198, the entirety of

5 which is incorporated herein by reference. The plastid transformation vector pCGN6146 is
designed by replacing the DNA sequence encoding for GUS from pCGN4276 (described
in USPN 5,576,198) with the coding sequence of aprotinin. The tobacco plastid
transformation construct pCGN6147 contains the same elements as pCGN6146 except
10 pCGN6147 contains the six 5' amino acids of the GUS encoding sequence ligated to the 5'
terminus of the aprotinin encoding sequence. The six amino acids of the 5' terminus of the
GUS nucleotide sequence are included to aid in the translation of the aprotinin protein.
15 The tobacco plastid transformation vector pCGN6156 is essentially the same as
pCGN4276 except the coding region of aprotinin is cloned to the 3' end of the GUS
20 coding sequence. Thus, pCGN6156 contains as operably linked the T7 promoter, a DNA
sequence encoding for GUS fused with the DNA sequence encoding for aprotinin and the
psbA 3' transcription termination sequence.

20 A plastid expression construct, pCGN6154, was constructed from pCGN4276 by
replacing the GUS coding sequence with the aprotinin protein operably linked to the 3'
25 terminus of the coding sequence of cytochrome *f* (petA) of the tobacco chloroplast. Thus,
pCGN6154 contains the T7 promoter sequence operably linked to the nucleotide sequence
of petA and aprotinin. The petA sequence is included to direct the expressed aprotinin
protein to the thylakoid.
30

20 **Example 2 Plant Transformation**

2A. *Nuclear Transformation*

35 Tobacco plants transformed to express the constructs pWRG4744 and pWRG4747
in the nucleus of a plant cell may be obtained as described by Horsch *et al.* (*Science* (1985)
227:1229-1232).

25 40 2B. *Plastid Transformation*

Tobacco plastids are transformed by particle gun delivery of microprojectiles as
described by Svab and Maliga (*Proc. Natl. Acad. Sci.* (1993) 90:913-917), and described
herein.

30 45 50 Dark green, round leaves are cut, preferably from the middle of the shoots, from 3-
6 week old *Nicotiana tabacum* cv. Havana which have been maintained *in vitro* on
hormone free MS medium (Murashige and Skoog, (1962) *Physiol Plant.* 15, 473-497)
supplemented with B5 vitamins in Phytatrays or sundae cups with a 16 hour photoperiod at

5 24°C. Each cut leaf is then placed adaxial side up on sterile filter paper over tobacco shoot
regeneration medium (TSO medium: MS salts, 1mg/l N^6 -benzyladenine, 0.1mg/l 1-
10 naphthaleneacetic acid, 1 mg/l thiamine, 100mg/l inositol, 7g/l agar pH 5.8 and 30g/l
 sucrose). Leaves are preferably placed in the center of the plate with as much contact with
15 the medium as possible. The plates are preferably prepared immediately prior to use, but
 may be prepared up to a day before transformation by particle bombardment by wrapping
 in plastic bags and storing at 24°C overnight.

15 Tungsten or gold particles are sterilized for use as microcarriers in bombardment
 experiments. Particles (50mg) are sterilized with 1 ml of 100% ethanol, and stored at
10 -20°C or -80°C. Immediately prior to use, particles are sedimented by centrifugation,
 washed with 2 to 3 washes of 1 ml sterile deionised distilled water, vortexed and
20 centrifuged between each wash. Washed particles are resuspended in 500 μ l 50% glycerol.

25 Sterilized particles are coated with DNA for transformation. Twenty-five
 microliter aliquots of sterilized particles are added to a 1.5 ml microfuge tube, and 5 μ g of
15 DNA of interest is added and mixed by tapping. Thirty-five microliters of a freshly
 prepared solution of 1.8M CaCl₂ and 30 mM spermidine is added to the particle/DNA
 mixture, mixed gently, and incubated at room temperature for 20 minutes. The coated
30 particles are sedimented by centrifuging briefly. The particles are washed twice by adding
 200 μ l 70% ethanol, mixing gently, and centrifuging briefly. The coated particles are
20 resuspended in 50 μ l of 100% ethanol and mixed gently. Five to ten microliters of coated
 particles are used for each bombardment.

35 Transformation by particle bombardment is carried out using the PDS 1000 Helium
 gun (Bio Rad, Richmond, CA) using a modified protocol described by the manufacturer.

40 Plates containing the leaf samples are placed on the second shelf from the bottom
25 of the vacuum chamber and bombarded using the 1100 p.s.i. rupture disk. After
 bombardment, petriplates containing the leaf samples are wrapped in plastic bags and
 incubated at 24°C for 48 hours.

45 After incubation, bombarded leaves are cut into approximately 0.5 cm² pieces and
 placed abaxial side up on TSO medium supplemented with 500 μ g/ml spectinomycin.

50 30 After 3 to 4 weeks on the selection medium, small, green spectinomycin resistant shoots
 will appear on the leaf tissue. These shoots will continue to grow on spectinomycin
 containing medium and are referred to as primary putative transformants.

- 5 When the primary putative transformants have developed 2 to 3 leaves, 2 small
pieces (approximately 0.5 cm²) are cut from each leaf and used for either selection or for a
second round of shoot regeneration. One piece is placed abaxial side up on plates
containing TSO medium supplemented with 500 µg/ml spectinomycin, and the other piece
10 5 is placed abaxial side up on TSO medium supplemented with 500 µg/ml each of
spectinomycin and streptomycin. Positive transformants are identified as the shoots which
form green callus on the TSO medium containing spectinomycin and streptomycin.
- 15 After 3 to 4 weeks, the tissue placed on TSO medium containing only
spectinomycin, which has been identified as positive on the TSO medium with
10 spectinomycin and streptomycin, will develop green shoots. Two to four shoots of each
positive transformant are selected and transferred to TSO medium supplemented with 500
20 µg/ml spectinomycin for generation of roots. Southern analysis is performed on 2 shoots
to confirm homoplasmy as described below. Shoots from homoplasmic events are
transferred to the greenhouse for seed production, while transformants which are not
25 15 homoplasmic are sent through a second round of regeneration on TSO medium with 500
µg/ml spectinomycin to attain homoplasmy.

30 **Example 3 Analysis of Transplastomic Tobacco Plants Transformed with Herbicide
Tolerance Constructs**

20 **3A. Southern Analysis**

35 Transformed plants selected for marker *aadA* marker gene expression are analyzed
to determine whether the entire plastid content of the plant has been transformed
(homoplasmic transformants). Typically, following two rounds of shoot formation and
spectinomycin selection, approximately 50% of the transgenic plantlets which are analyzed
40 25 are homoplasmic, as determined by Southern blot analysis of plastid DNA. Homoplasmic
plantlets are selected for further cultivation.

45 Genomic DNA is isolated from transformed tobacco plants, electrophoresed, and
transferred to filters as described in Svab *et al.* ((1993), *Proc Natl Acad Sci*, 90:913-917).

Homoplasmic tobacco plants transformed to express CP4 EPSPS in plastids were
50 30 identified using a probe prepared from a 2.4 kb *Eco*RI/*Eco*RV fragment from the vector
pOVZ2 (similar to pOVZ15 described in Zoubenko, *et al.* 1994, *supra*). The 2.4 kb probe
fragment encompasses part of the targeting sequence.

5 Results of the Southern hybridizations identified 3 homoplasmic lines from
tobacco transformed with the constructs pMON30123 and pMON30130 and 1 line from
tobacco transformed with pMON38773 for further analysis.

10 The complete disappearance of the 3.27 Kb native tobacco *Bam*HI fragment in the
5 lines 30123-19-1A, 30123-23-2A, 30123-18-1B, 30130-51-2A, 30130-51-2P, 30130-57-
1P, and 38773-6 with a probe covering the region of integration, and the appearance of
expected sized bands for the inserted DNA fragments in those transformants, 5.14 kb and
15 0.9 kb, establishes that the transformed plants are homoplasmic for the intended
constructs.

10 Results of the Southern hybridizations identified 3 homoplasmic lines from
20 tobacco transformed with pCGN5177, lines 74-1B-P, 74-2 and 74-7.

20 Transplastomic 5175 and 6114 tobacco lines were analyzed by Southern
hybridization for homoplasmy as described above. Results of the Southern hybridizations
identified 4 homoplasmic lines from tobacco transformed with pCGN6114.

25 Results from hybridizations of 5175 transplastomic tobacco lines identified one
15 line, 76-4A-F, as homoplasmic, and a second line as 95% homoplasmic.

30 Homoplasmic tobacco plants transformed to express BxN/AHAS in plastids were
identified using Southern hybridizations as described above.

30 Results of the Southern hybridizations identified 14 homoplasmic lines from
20 tobacco transformed with pCGN5026. The filters were reprobed with a BxN gene
fragment, and 21 lines were found to contain BxN, 14 lines of which were homoplasmic.

35 3B. *Northern Analysis*
40 In order to determine the level of transcription of the EPSPS, BxN or AHAS
25 mRNA expressed in the transplastomic tobacco plants, Northern blot hybridizations were
40 performed with total RNA isolated from each of the lines identified. Total RNA was
isolated using TRIzol reagent (Gibco-BRL Life Technologies, Gaithersburg, MD)
45 according to the manufacturers protocol. Total RNA, 2 μ g, was separated on a denaturing
agarose gel and transferred to nylon membrane (Maniatis *et al.*, 1989, *supra*). Radioactive
50 probes for hybridizations were prepared using random primer labeled (using Random
Primer labeling kit from Boehringer Mannheim) CP4 EPSPS, phytoene desaturase, BxN,
or AHAS fragments and hybridizations were carried out in 2x SSPE (Maniatis, *et al.*,
1989,*supra*), at 60°C. Filters were stripped and reprobed with a plastid 16S ribosomal

5 RNA gene probe (from pPRV112A, Zoubenko, *et al.*, 1994, *supra*) to confirm
homogenous loading of RNA on the filter.

10 Results of the Northern hybridizations performed with EPSPS probes demonstrate
that all seven (7) lines examined express CP4 EPSPS mRNA. Hybridizations performed
15 with the 16S ribosome probe confirm that denaturing gels were loaded with similar
amounts of total RNA for each sample. Furthermore, transplastomic tobacco lines
expressing EPSPS from the *Prrn/rbcL(RBS)* (pMON30123) regulatory elements express
EPSPS mRNA to higher levels than tobacco plants homoplasmic for EPSPS controlled by
the *Prrn/G10L* (pMON38773) promoter/RBS sequences.

20 10 Results of Northern hybridizations performed with BXN, AHAS and *crlI* probes
demonstrates that all homoplasmic 5026, 5175, and 5177 tobacco lines expressed *crlI*,
BXN and/or AHAS mRNA.

3C. Western Blot Analysis of Tobacco CP4 EPSPS

25 15 To determine the expression of the EPSPS, Western blot analysis was performed
on a single line from each construct, pMON30123, pMON30130, and pMON38773.

30 20 Total soluble protein was extracted from frozen leaf tissue by grinding 250 mg
tissue in 250µl of PBS buffer (1 mM KH₂PO₄, Na₂HPO₄, 0.137M NaCl, 2.7 mM KCl pH
7.0) containing protease inhibitors. The homogenate is centrifuged for 5 minutes, and the
25 supernatant is transferred to a fresh tube. The concentration of the protein in the
supernatant is determined using a protein concentration assay (BioRad, Richmond, CA).

35 35 Extracted total protein is electrophoresed on a 4-20% SDS-PAGE gel (Sigma, St
Louis, MO), and transferred to PVDF membrane in 1x SDS-PAGE buffer (Maniatis *et al.*
1989, Cold Spring Harbor Press). Standards of quantitated purified CP4 EPSPS protein
40 25 were used to quantify the expression of the CP4 EPSPS as expressed in the plant plastid.

45 40 Western hybridizations are performed as described in Staub and Maliga (1993)
EMBO Journal, 12(2) 601-606, except using antibodies raised to EPSPS. PVDF
membranes containing the transferred electrophoresed protein were incubated in a
blocking solution of PBS buffer containing 0.05% Tween-20 (PBS-T) and 5% milk
30 45 overnight at 4°C. The membranes are then incubated in a solution of PBS-T containing
1% milk and a primary antibody raised in goats to the CP4 EPSPS for 2 hours at room
temperature. The membranes are washed three times in a solution of PBS-T containing
50 50

5 0.1% milk, each wash for 5 minutes at room temperature. The membranes are then
incubated in a solution of PBS-T containing 1% milk and sheep anti-goat antibody for 1
hour at room temperature, and washed again in PBS-T containing 0.1% milk, three times
10 for 10 minutes at room temperature. A final wash using only PBS-T is performed before
10 developing the membranes using a nonradioactive detection kit (ECL, Amersham).

15 **Table 2**

Construct Number	Event Number	% Total Soluble Protein
pMON30123	T18-23-2A	0.001
pMON30130	T18-51-2P	0.002
pMON38773	9706-6-1	0.2

10 The results listed in Table 2 demonstrate that significant increases in the level of
30 EPSPS protein may be obtained from plants transformed to express EPSPS from the
P_{rrn}/G10L promoter. These results demonstrate that EPSPS expression driven by the
P_{rrn}/rbcLRBS regulatory sequences may produce approximately 0.001% of the total
35 soluble protein as EPSPS, while in plants expressing EPSPS from the P_{rrn}/G10L
regulatory sequences express 0.2% of the total soluble protein as EPSPS. Subsequent lines
have demonstrated total soluble protein of about 1% EPSPS when expressed from the
40 P_{rrn}/G10L regulatory sequences. These results, taken together with the results of the
Northern hybridizations above, indicate that more efficient translation may be obtained
from the G10L ribosome binding site.

45 Western immunoblot hybridization were also performed on 2 homoplasmic 5026
tobacco lines as described above, using antibodies raised against bromoxynil. The results
of Western immunoblot analysis of total soluble protein extracted from tobacco lines
transformed with pCGN5026 demonstrated that both homoplasmic lines produced nitrilase
50 protein.

5 Western immunoblot analysis was performed as described above from total protein
extracted from tobacco lines transformed with pCGN6114 and pCGN5197.

10 The results of the analysis demonstrated that bromoxynil was produced in 6114
tobacco lines ranging from 1% to 2% of the total soluble leaf protein.

15 5 The results of the Western analysis of the 20 5197 tobacco lines demonstrated that
bromoxynil and Bt were both produced as 1% of the total soluble leaf protein.

15 3D. Analysis of EPSPS Enzyme Activity

20 10 The EPSPS enzyme activity in transplastomic tobacco plants containing the plastid
expression vector pMON38773 was determined using a high pressure liquid
chromatography (HPLC) assay.

25 20 Methods for the analysis of EPSPS enzyme activity are described in Padgett *et al.* (*J.
Biol. Chem.* (1988)263:1798-1802 and *Arch. Biochem. Biophys.* (1987)258:564-573) and
Wibbenmeyer *et al.* (*Biochem. Biophys. Res. Commun.* (1988)153:760-766).

15 15 The results are summarized in Table 3 below.

Table 3

<u>Nuclear</u>	<u>Nuclear</u>	<u>Chloroplast</u>
Enzymatic Activity	% Total Plants	38773-6
Range	In Range	
1-3.7 μ mol/mg	1%	,
>0.1 μ mol/mg	16%	
>10 nmol/mg	55%	16.39 nmol/mg
>1 nmol/mg	32%	
0 nmol/mg	3%	

20 45 These results demonstrate that EPSPS expression in plastids produces active EPSPS
enzyme.

50

5 *3E. Analysis for Glyphosate Tolerance*

A transplastomic tobacco line homoplasmic for the construct pMON38773 was tested *in vitro* to determine the highest level of glyphosate tolerance. Explant tissue was prepared from leaf pieces of nontransgenic wild type tobacco control, *Havanna*, plants and 10 the homoplasmic tobacco line 38773-6 and cultured for regeneration of shoots on TSO medium (described above) supplemented with glyphosate levels of 50 μ M, 75 μ M, 100 μ M, 150 μ M and 200 μ M. The results are summarized in Table 4 below. The number of 15 explants producing shoots was determined at 3 weeks and 6 weeks after explant preparation and culturing on glyphosate containing medium.

10

20 Table 4

Glyphosate Level (μ M)	Total Number Explants	Number Regenerating 3 Weeks	Number Regenerating 6 Weeks	% Explant Regeneration
Wild Type				
50	10	0	0	0
75	10	0	0	0
100	10	0	0	0
150	10	0	0	0
200	10	0	0	0
38773-6				
50	8	5	8	100
75	18	14	18	100
100	17	12	15	88
150	18	10	16	89
200	16	8	15	86

The above results demonstrate that at all levels of glyphosate examined, shoots 50 15 regenerated from explants prepared from a tobacco line homoplasmic for pMON38773,

5 while no shoots regenerated from explants prepared from nontransformed control plants.
 These results suggest that tobacco plants expressing EPSPS in plastids demonstrate
 tolerance to glyphosate levels of at least 200 μ M.

10 Additional transplastomic lines were tested *in vitro* for glyphosate tolerance as
 5 described above. The results are shown in Table 5.

TABLE 5

15 **Summary of tobacco plastid transformation experiments with various constructs
 containing EPSPS genes.**

	Construct	Spec/strep (+)	No. of shoots Gly 50 uM(+)
10	pMON38766 (Wild)	1	0
20	pMON38766 (T7)	6	0
15	pMON38773 (Wild)	9	5 (1)
25	pMON38797 (Wild)	2	0
	pMON38798	6	6
30	pMON38793	8	0
20	pMON38796	4	0
	pMON45201	9	3
	pMON45204	12	*
35	(No. of shoots positive at 1 mM glyphosate)		
25			

40 These results demonstrate that these transplastomic lines show tolerance to
 glyphosate. The numbers in parentheses are the number of shoots resistant to selection at
 1 mM glyphosate. Thus, as can be seen in table 5, tobacco lines are generated that are
 45 tolerant of selection at 1 mM glyphosate.

50 Homoplasmic tobacco plants of the line 38773-6 are sprayed with glyphosate using
 a track sprayer at concentrations corresponding to 0oz/acre, 16oz/acre, 32oz/acre and
 64oz/acre to test for whole plant tolerance. Plant height was measured before and after

5 spraying with glyphosate. The vegetative injury data was collected two weeks after spraying, while the reproductive injury data was collected at plant maturity.

Initial results indicate that homoplasmic tobacco lines sprayed are tolerant of 10 glyphosate at the concentration of 16oz/acre as demonstrated in the vegetative tissue injury 5 (Table 6). As can be seen in Table 5 transplastomic lines were generated which demonstrated a good level of glyphosate tolerance at 32oz/Acre. In subsequent experiments with additional transformed lines, transplastomic lines have shown tolerance 15 to glyphosate at a level of 64oz/Acre.

Tolerance is characterized by the continued growth and greening of tissues sprayed 10 with glyphosate. However, as the concentration of glyphosate applied increased, there was a corresponding increase in the level of vegetative injury. In contrast, nontransformed 20 control plants which were highly susceptible to glyphosate concentrations as low as 16 oz/Acre.

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Table 6

	Plant No.	Construct	Roundup rate (oz/A)	Plant height (cm) before spray	Plant height (cm) after spray	Vegetative injury	Fertility rating
10	1	38773	0	12.2	30.5	0	0
15	2	38773	0	13.6	34.0	0	0
20	3	38773	0	8.6	23.8	0	0
25	4	38773	0	8.6	26.2	0	0
30	5	38773	0	7.8	28.8	0	0
35	6	38773	0	12.8	31.5	0	0
40	7	38773	0	12.2	31.6	0	0
45	8	38773	0	11.6	35.5	0	0
50	9	38773	16	9.0	29.0	1	0
	10	38773	16	14.4	31.0	0	0
	11	38773	16	13.4	32.0	0	0
	12	38773	16	13.2	30.0	0	0
	13	38773	16	14.2	30.5	0	1
	14	38773	16	14.0	33.0	0	0
	15	38773	16	13.2	30.2	0	0
	16	38773	16	14.9	30.4	0	0
	17	38773	32	12.0	26.5	2	4
	18	38773	32	11.6	25.4	1	1
	19	38773	32	9.4	22.0	1	3
	20	38773	32	11.2	23.0	2	4
	21	38773	32	13.8	25.8	1	2
	22	38773	32	12.4	23.0	1	4
	23	38773	32	10.2	19.0	2	4
	24	38773	32	13.8	23.2	2	3
	26	38773	64	11.8	20.0	2	5
	27	38773	64	13.0	22.0	2	5
	28	38773	64	12.2	18.0	3	5

5	29	38773	64	15.8	23.0	2	5
	30	38773	64	10.4	17.5	2	5
	32	38773	64	15.0	18.5	2	5
10	33	38773	64	13.8	21.8	2	5
	34	38773	64	13.6	19.0	3	5
	35	38773	64	10.8	16.0	3	5
15	36	Wild type	0	21.0	40.6	0	0
	37	Wild type	0	16.0	38.0	0	0
	38	Wild type	0	15.0	34.6	0	0
20	39	Wild type	0	17.6	32.2	0	0
	40	Wild type	0	15.0	31.6	0	0
	41	Wild type	0	14.0	32.0	0	0
	42	Wild type	16	10.0	11.8	3	5
25	43	Wild type	16	8.0	10.0	3	5
	44	Wild type	16	8.6	11.0	3	5
	45	Wild type	16	8.0	14.0	3	5
30	46	Wild type	16	9.8	11.0	3	5
	47	Wild type	16	10.4	14.0	3	5
	48	Wild type	32	10.8	13.2	3	5
35	49	Wild type	32	9.0	13.0	3	5
	50	Wild type	32	8.0	10.2	3	5
	51	Wild type	32	11.0	14.0	4	5
40	52	Wild type	32	9.8	13.0	3	5
	53	Wild type	32	8.0	10.8	4	5
	54	Wild type	64	7.5	8.6	4	5
	55	Wild type	64	11.2	12.5	4	5
45	56	Wild type	64	10.2	12.8	4	5
	57	Wild type	64	11.5	13.0	4	5
	58	Wild type	64	13.0	15.0	4	5
	59	Wild type	64	9.8	11.2	4	5

Vegetative injuries:

- 5 0=normal plant.
1=slight chlorosis of new leaves and stunting
2=severe chlorosis of new leaves, malformation of new leaves, and severe
stunting
10 3=dying plant
4=dead plant

- 15 Fertility ratings:
0 = Fertile, no delay in maturity, lots of seed
1 = Some abortion, slight delay in seed set, seed
2 = Significant abortion, significant delay in seed set, some seed
20 3 = Very severe abortion, immature seed pots, a few seed
4 = malformed flowers; if flowered, extreme delay in flowering and no seed
produced
25 5=dead plant

3F. BT/BXN Analysis

30 Homoplasmic tobacco plants of the lines 5175 and 5197 are sprayed with Buctril
herbicide at a concentration of 4% to test for whole plant tolerance.

Results of the spray test with Buctril demonstrated that all 5197 lines expressing
5 *bxn* were completely resistant when sprayed with a solution containing 4% Buctril
herbicide.
35

Two lines out of six 5175 lines tested were completely resistant to the herbicide
when sprayed with a 4% solution containing Buctril.

40 10 *3G. Norflurazon Resistance Analysis*

An experiment was set up to determine the efficacy of the Crt I trait with respect to
resistance to the herbicide Norflurazon. Three 5177 transformed lines, 74-1B-P, 74-2-A,
45 and 74-7-C and three control lines were planted. Plants were grown for seven weeks and
then watered with a 3 μ M Norflurazon solution. Plants negative for the presence of the *crtI*
15 plastid-borne gene were bleached by Norflurazon treatment, positive plants stayed green
and continued to grow.
50

5 The results show that the three homoplastic 5177 tobacco lines were resistant to
the 3 μ M Norflurazon solution, while the control plants were all susceptible to the solution
(Table 7).

10

5

Table 7

15

20

Line	Control/Transgenic	Result
Xanthi	Control	Susceptible
2560A Xanthi	Control	Susceptible
75-5D-A	Control	Susceptible
74-1B-P	homoplastic	Resistant
74-2-A	homoplastic	Resistant
74-7-C	homoplastic	Resistant

25

Example 4 Analysis of hGH Transgenic Tobacco Plants**4A. Southern Analysis**

10 Transformed plants selected for *aadA* marker gene expression are analyzed to
determine whether the entire plastid content of the plant has been transformed
30 (homoplastic transformants). Homoplastic plants are selected using Southern
hybridization for further cultivation.

35

Genomic DNA is isolated from transformed tobacco plants, electrophoresed, and
15 transferred to filters as described in Svab *et al.* ((1993), *Proc Natl Acad Sci*, 90:913-917).

40

Homoplastic tobacco plants transformed to express hGH were identified using a
probe prepared from a 2.4 kb *Eco*RI/*Eco*RV fragment from the vector pOVZ2 (similar to
pOVZ15 described in Zoubenko, *et al.* 1994, *supra*). The 2.4 kb probe fragment
encompasses part of the targeting sequence.

45

20 The complete disappearance of the 3.27 Kb native tobacco *Bam*HI fragment in the
lines with a probe covering the region of integration, and the appearance of the expected
size band for the inserted DNA fragments in those transformants, 5.6 kb, establishes that
the transformed plants are homoplastic for the intended constructs.

50

55

4B. Protein Expression Analysis

5

Homoplasmic tobacco lines expressing hGH and nuclear tobacco transformants are used to determine the expression of the hGH protein. Western blot analysis was performed on tobacco lines containing constructs pWRG4838, pMON38755 and pMON38794 for 10 plastid expression and an ELISA assay was used for transgenic tobacco lines containing pWRG4744 and pWRG4747 for nuclear expression of hGH.

10

Total protein extractions and western blot procedures were performed as described above, with the exception of the primary antibody was raised against hGH.

15

10

Table 8
Expression Levels of hGH in Tobacco Nuclear Genome and Plastid genome

20

Construct	Expression	Expression Level % Total Soluble Protein
pWRG4744	nuclear	0.002-0.125%
pWRG4747	nuclear	0.002-0.025%
pWRG4838	plastid	0.2%
pMON38755	plastid	1.0%
pMON38794	plastid	7.0%

25

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Results of the Western analysis (Table 8) demonstrates that hGH expressed in plastids of plant cells accumulates to significantly higher levels than hGH expressed in the nucleus and targeted to either the cytoplasm or plastid of plant cells. Tobacco plants transformed to express hGH in the nucleus accumulated hGH levels of 0.002% (cytoplasmic targeted) to 0.025% (plastid targeted) of total soluble leaf protein, while tobacco plants expressing hGH in the plastid accumulated hGH levels of 0.2% to 7.0% of the total soluble leaf protein as hGH. Furthermore, homoplasmic tobacco plants expressing hGH directed from the *Prrn/G10L* regulatory sequences accumulate 35 fold higher levels of hGH than homoplasmic tobacco plants expressing hGH directed from the *PpsbA* promoter sequence. The higher level of expression may be due to the strong *Prrn* promoter and/or to enhanced translation of the fusion gene mediated by the gene 10 leader rbs region. Leaves of different ages had varied hGH accumulation patterns, with mature

5 and old leaves having similar levels and younger leaves much less hGH. This is consistent with the lower chloroplast number in young leaves.

10 Interestingly, both ubiquitin-hGH and processed hGH accumulated in the post-harvest extracts of the Nt-38755 and Nt-38794 lines. Ubiquitin processing was often observed at >50% of total hST protein species, depending on extraction conditions. This result confirms the utility of the fusion protein approach in chloroplast-expressed proteins. The appearance of an extra band observed in the Nt-4838 sample is consistent with an hGH dimer.

15 For comparison of expression systems in plants, nuclear transgenic plants were generated that express hGH from two different sets of expression signals. The wrg4747 and wrg4776 constructs expresses hGH using the strong Figwort Mosaic Virus promoter or the Cauliflower Mosaic Virus 35S promoter, respectively. The wrg4747 construct employs a chloroplast transit peptide to post-translationally target hGH to chloroplasts (FMV::CTP-hGH), whereas the wrg4776 construct targets the hGH through the endoplasmic reticulum (ER) to the secretory pathway (35S::ER-hST). Transgenic lines for both constructs were obtained through particle bombardment. Expression of hST was quantitated by ELISA assay and shown to be less than 0.025% tsp. This level of expression is at least 300-fold lower than the pMON38794 lines, proving the feasibility of the chloroplast expression system for the potential production of hST.

20

30 *4C. Characterization of hGH Protein Expressed in the Plastid*

35 In order to determine whether the hGH expressed from plastids was properly processed, experiments were performed to determine correct folding and bioactivity.

40 Two bottom leaves of transplastomic tobacco lines containing pMON38794 were used to extract and purify hGH. Large veins were removed from the excised leaves, and the leaf tissue was cut into small sections (approximately 0.5 cm²). The leaf pieces were flash frozen in liquid nitrogen and ground to a fine powder in a chilled mortar and pestle. Ten grams of frozen, ground leaf tissue was added to ice cold 100 mM Tris base solution (30 ml) and mixed vigorously by vortexing for 5 minutes. The solution was filtered 45 through a single layer of cheese cloth.

50 From the filtered solution, three separate samples were prepared. The first sample was prepared by centrifuging 4 ml of the filtrate for 1 minute at 16,000 rpm. The centrifugate was aliquoted into 1 ml vials and frozen in dry ice. The remaining filtrate was

5 centrifuged for 10 minutes at 4800 rpm, and several 0.5 ml aliquots were frozen as above
for the second sample. To the remaining centrifuged filtrate (approximately 25 ml), 200 μ l
of glacial acetic acid was added to lower the pH from 8.2 to 4.56. The solution was
centrifuged at 4800 rpm for 30 minutes, and the supernatant was frozen over dry ice for the
10 5 third sample.

15 Total soluble protein (TSP, Table 9) was calculated in these samples by standard
protein assay procedures (Maniatis,), and the percent purity of hGH was calculated based
on results from Western blot analysis using known concentrations of starting material.

10

Table 9

Sample ID	TSP mg/mL	GP2000 mg/L	% Purity
Filtered Extract immediately centrifuged and frozen	6.3	28	0.45 %
Filtered extract centrifuged at 4800 rpm for 10 min and frozen	6.4	28	0.45
pH adjusted and centrifuged extract	0.75	21	2.8%

The pH adjusted and centrifuged extract was purified by Reverse Phase-HPLC

35 15 (RP-HPLC) for electrospray mass spectrometry and amino-terminal amino acid
sequencing. RP-HPLC was performed using a Perkin-Elmer series 200 pump and
autosampler and a Vydac C8 (250 by 4.6 mm) RP-HPLC column. 750 microliters of
40 sample was loaded onto the column equilibrated with 20 mM trifluoroacetic acid (TFA)
and 50 % acetonitrile. After loading, the column was washed for 2 minutes with 50 %
20 acetonitrile, 20 mM TFA followed by a 2% linear acetonitrile gradient over 10 minutes
followed by a 10 % acetonitrile gradient over 1 minute. The flow rate was a constant 1.5
45 ml/minute with the column eluate monitored at 278 nm with a Perkin-Elmer 785 detector.
Data was collected and analyzed with a PE-Nelson Turbochrom data system.

50 25 The results of the RP-HPLC analysis are shown in figure 3. Peak I (tallest peak)
has the retention time expected for properly folded, native 22 kDa GP2000. This peak was

5 collected and dried down in a Savant Speed-Vac for amino terminal sequencing and
electrospray mass spectrometry.

10 Electrospray ionization mass spectrometry (MS) analysis used a Micromass Q-ToF
electrospray time-of-flight mass spectrometer. The samples were prepared by
5 resuspending in 50% methanol + 2% acetic acid, and infused into the source of the mass
spectrometer at a rate of 4mL/min. The raw data shown in Figure 4 shows a series of ions
corresponding to the specie(s) present in the sample with varying numbers of protons
15 attached. The axes of this spectrum are intensity versus mass-to-charge ratio of the
specie(s) present. A deconvolution algorithm is used to convert this series of multiply
10 charged ions into a molecular weight spectrum.

20 The results of the mass spectrometry of the RP-HPLC peak 1 shows 4 major protein
species of different molecular mass. The 21,997 kDa species represents the predicted
mass of hGH with the predicted N-terminal Phe removed by over-cleavage of the
Ubiquitin protease with an N-terminal proline residue (P-hGH). The 22,124 kDa species
25 represents the predicted mass of properly processed, correct amino acid sequence of hGH
having the N-terminal phenylalanine (F-hGH). The 22,507 kDa and 22,664 kDa species
are thought to represent an hGH with the N-terminal Phe and hGH which has been
modified during plant extraction procedures, respectively. The calculated molecular mass
30 of the proteins suggests that the hGH expressed from the plastid is properly folded (i.e. the
correct disulfide bonds are created).

35 Equivalent mobility to refolded *E. coli* produced protein indicates formation of the
two disulfide bonds and proper folding of the chloroplast derived hGH. This result was
surprising because of the prokaryotic nature of chloroplasts. There are no known, plastid-
expressed proteins that have disulfide bonds. However, nuclear-encoded, imported
40 enzymes can be activated by disulfide bond oxidation/reduction cycles, presumably using
the chloroplast thioredoxin system (Jacquot, *et al.* (1997) *New Phytol.* 136:543-570) or a
recently discovered chloroplast protein disulfide isomerase (Kim and Mayfield (1997)
Science 278:1954-1957). This result suggests that the prokaryotic organelle has the
machinery needed to fold complex eukaryotic proteins in the soluble chloroplast stroma
45 compartment. This is distinct from *E. coli*, where recombinant proteins tend to accumulate
within inclusion bodies, and then require solubilization and refolding.

50 Amino terminal sequencing was done by standard Edman degradation, and
confirmed the N-terminal sequences discussed above.

5

4D. Bioactivity of hGH Expressed in Plant Plastids

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Bioactivity of the pH adjusted and centrifuged extract was tested using cells from an Nb2 cell line. These cells proliferate in the presence of growth hormone and other estrogenic type compounds. The assay involves putting various concentrations of growth hormone-containing extract into a 96 well plate. Then a constant amount of cells are added to each well. The plate is incubated for 48hrs and then a reagent called MTS is added. Metabolizing cells take up the MTS and convert it to a blue colored substance. The more cells there are the more blue color in the well. The blue color is measured using a spectrophotometer. The number of cells should be proportional to the concentration of growth hormone in the media. At some high concentration one expects that the cells will become saturated with growth hormone and that the dose response will level off. At very low hGH concentrations essentially no enhanced growth is seen. A sigmoidal shape graph is expected to be produced graphing the cell number (or absorbance) versus hGH concentration graph.

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Proper disulfide pairing in the chloroplast hGH implies that the protein should be biologically active. To test this hypothesis *in vitro*, a rat lymphoma cell line, Nb2, that proliferates in the presence of somatotropin (hGH) and other estrogenic type compounds was employed. Proliferation of this cell line is proportional to the amount of somatotropin in the culture medium, until saturation is reached. The ion exchange column eluate from transplastomic Nt-4838 and Nt-38794 plants or identically treated wild-type plants was added to the Nb2 cell culture medium. As control, *E. coli* produced, refolded hGH was used. The wild-type plant extract showed no activity in this assay, indicating that there is no endogenous plant compound capable of stimulating growth of the Nb2 cell line. In contrast, the Nt-4838 and Nt-38794 extracts both stimulated proliferation of the cell line to an equal extent as the positive controls: either wild-type plant extract that had been spiked with purified *E. coli* hGH or the pure hGH alone.

The Nb2 cell results show that the chloroplast derived hGH is biologically active.

Previous studies of recombinant somatotropin produced in *E. coli* showed equivalent pharmacokinetics of the protein with either an N-terminal methionine or phenylalanine (Moore, et al. (1988) *Endocrinology* 122:2920-2926). In this study, ubiquitin cleavage of the fusion protein in Nt-38794 lines generated predominantly P-hST, suggesting that this species is also bioactive. The hST from Nt-4838 extracts was also characterized. Amino

acid analysis indicated >95% protein species with alanine at the N-terminus. This result
5 suggests that a methionine aminopeptidase activity generated the alanine-hST, which is
also bioactive. A similar aminopeptidase activity exists in *E. coli* (Meinnel, *et al.* (1993)
Biochimie 75:1061-1075). This finding in plastids may be exploited in the future as an
10 alternative means to generate a non-methionine N-terminus.

15 The results of the bioactivity assay (Figure 5) demonstrates that the hGH expressed
from a plant plastid has a sigmoidal shape when graphed as absorbance versus hGH
concentration.

10 **Example 5 Analysis of Aprotinin Transplastomic Tobacco Plants**

20 **5A. Western Analysis of Aprotinin Expression in Plastids**

Homoplastic tobacco lines expressing are used to determine the
expression of the aprotinin protein. Western blot analysis was performed on tobacco lines
25 containing constructs pCGN6146, pCGN6147, pCGN6154 and pCGN6156 for plastid
expression of aprotinin.

30 Total protein extractions and western blot procedures were performed as described
above, with the exception of the primary antibody was raised against aprotinin.

35 The results of the Western analysis is shown in Figure 6. These results indicate
that aprotinin is expressed from the T7 polymerase promoter when the aprotinin coding
sequence is fused with either the PetA or full length GUS gene. Furthermore, these results
indicate that the petA sequence efficiently targets the aprotinin protein to the plant cell
thylakoid.

40 All publications and patent applications mentioned in this specification are
25 indicative of the level of skill of those skilled in the art to which this invention pertains.
All publications and patent applications are herein incorporated by reference to the same
extent as if each individual publication or patent application was specifically and
individually indicated to be incorporated by reference.

45 Although the foregoing invention has been described in some detail by way of
30 illustration and example for purposes of clarity of understanding, it will be obvious that
certain changes and modifications may be practiced within the scope of the appended
claim.

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Claims

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CLAIMS

What is claimed is:

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1. A construct comprising the following components in the 5' to 3' direction of transcription:

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a) a promoter functional in a plant plastid;

b) a DNA sequence encoding a peptide derived from an eukaryotic organism; and

10

c) a transcription termination region;

wherein said eukaryotic peptide is other than a peptide of a plant plastid.

20

2. The construct according to Claim 1, wherein said construct further comprises

(d) a gene encoding a selectable marker for selection of plant cells comprising a plastid
15 expressing said marker and (e) DNA regions of homology to the genome of said plastid,
wherein said regions of homology in (e) flank components (a), (b), (c) and (d).

25

3. The construct according to Claim 1, wherein said construct further comprises

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(f) a ribosome binding site joined to said promoter component (a).

20

4. The construct according to Claim 3, wherein said ribosome binding site (f) is
35 from a leader sequence selected from the group consisting of sites derived from plastid,
bacterial or bacteriophage leader sequences.

40

25

5. The construct according to Claim 4, wherein said ribosome binding site is
selected from the group consisting of the binding site of the gene 10 leader and the
rbcLRBS site.

45

30

6. The construct according to Claim 1 wherein said DNA sequence encodes a plant
nuclear peptide.

50

7. The construct according to Claim 6 wherein said plant nuclear peptide is a
carbon cycle gene.

5

8. The construct according to Claim 7 wherein said a carbon cycle gene is selected from the group consisting of fructose 1,6 bisphosphatase aldolase and seduheptulose bisphosphatase,

10

5

9. The construct according to Claim 6 wherein said plant nuclear peptide is an antifungal peptide.

15

10. The construct according to Claim 1 wherein said DNA sequence encodes a mammalian peptide.

20

11. The construct according to Claim 10 wherein said mammalian peptide is a protein selected from the group consisting of interferons, monoclonal antibodies, hematopoietic agents, pituitary hormones, thyroid hormones, hypothalamic hormones, albumins and pancreatic hormones.

25

12. The construct according to Claim 11 wherein said mammalian peptide is the pancreatic hormone insulin.

30

20 13. The construct according to Claim 11 wherein said mammalian peptide is a pituitary hormone selected from the group consisting of somatomammotrophic hormones, gonadotropic hormones, thyrotropic hormones and chorticotrophic hormones.

40

25 14. The construct according to Claim 13 wherein said pituitary hormone is a gonadotropic hormone selected from the group consisting of chorionic gonadotropins, luteinizing hormones and follicle stimulating hormones.

45

30 15. The construct according to Claim 13 wherein said pituitary hormone is a somatomammotrophic hormone selected from the group consisting of prolactin and growth hormones.

50

16. The construct according to Claim 15 wherein said somatomammotrophic hormone is the growth hormone bGH.

5

17. The construct according to Claim 15 wherein said somatomammotrophic hormone is the growth hormone hGH.

10

5 18. The construct according to Claim 15 wherein said somatomammotrophic hormone is the prolactin hormone pBL.

15

19. The construct according to Claim 11 wherein said mammalian peptide is a hematopoietic agent selected from the group consisting of erythropoietins, interleukins, and colony stimulating factors.

20

20. The construct according to Claim 19 wherein said mammalian peptide is the colony stimulating factor G-CSF.

25

15 21. The construct according to Claim 11 wherein said monoclonal antibody is a single-chain F variable antibody.

30

22. The construct according to Claim 10 wherein said mammalian peptide is the non-enzymatic coagulation protein selected from the group consisting of factor V and factor VIII cofactor proteins.

35

23. The construct according to Claim 10 wherein said mammalian peptide is a proteinase inhibitor.

40

25 24. The construct according to Claim 10 wherein proteinase inhibitor is aprotinin.

45

25. The construct according to Claim 2 wherein said selectable marker is a selected from the group of aadA, spectinomycin resistance, streptomycin resistance kanamycin resistance and a glyphosate tolerance gene.

30

26. The construct according to Claim 1 wherein said DNA encoding sequence is the native encoding sequence to said gene.

50

5

27. The construct according to Claim 1 wherein said DNA encoding sequence is a synthetic encoding sequence to said gene.

10

5 28. A plant cell plastid containing the construct according to Claim 1.

15

29. A plant, plant seed, plant cell or progeny thereof containing a plant plastid according to Claim 29.

20

10 30. A method for producing a protein in a plant cell, wherein said method comprises transforming plastids of said plant cell with a construct comprising the following as operably joined components in the 5' to 3' direction of transcription:

25

(a) a promoter functional in a plant plastid;

(b) a DNA sequence encoding a peptide of an eukaryotic cell other than a peptide

15 of a plant plastid; and

(c) a transcription termination region.,

and growing plant cells comprising said transformed plastids under conditions wherein said DNA encoding sequence is expressed to produce said eukaryotic peptide in 30 said plastid.

20

31. The method according to Claim 30, wherein said construct further comprises

35

(d) a gene encoding a selectable marker for selection of plant cells comprising a plastid expressing said marker and (e) DNA regions of homology to the genome of said plastid, wherein said regions of homology in (e) flank components (a), (b), (c) and (d).

25

40

32. The method according to Claim 30, wherein said construct further comprises

(f) a ribosome binding site joined to said promoter component (a).

45

33. The method according to Claim 32, wherein said ribosome binding site (f) is from a leader sequence selected from the group consisting of sites derived from plastid, bacterial or bacteriophage leader sequences.

50

5 34. The method according to Claim 33, wherein said ribosome binding site is selected from the group consisting of the binding site of the gene 10 leader and the rbcLRBS site.

10 5 35. A plant cell produced according to the method of Claim 31 and comprising greater than about 0.01 % of total soluble protein as said eukaryotic peptide.

15 36. A plant cell according to Claim 31 and comprising greater than about 0.2 % of total soluble protein said eukaryotic peptide.

10 37. A plant cell according to Claim 31 and comprising greater than about 1 % of total soluble protein said eukaryotic peptide.

15 38. A plant cell according to Claim 31 and comprising 7 % or more of total soluble protein as said eukaryotic peptide.

30 39. A plant cell according to Claim 30 wherein said selectable marker comprises a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase.

20 40. A plant cell having a transformed plastid produced according to the method of Claim 30.

35 41. A plant, plant seed or plant part comprising a plant cell according to Claim 41.

25 42. The method according to Claim 30, wherein said construct further comprises (g) a coding sequence to a secondary protein fused to said DNA sequence encoding a peptide of an eukaryotic cell in (b).

45 43. The method according to Claim 42, wherein secondary protein is the thylakoid-targetting terminus of cytochrome f.

50 44. The method according to Claim 42, wherein secondary protein is the cleavable ubiquitin N-terminus.

5

45. The method according to Claim 44 whereby said cleavable ubiquitin N-terminus fusion is cleaved from said eukaryotic peptide by the step of harvesting said plant cells and exposing the contents of said transformed plastid to the cytosol of said plant cell.

10

5

46. The method according to Claim 45 whereby expression of said eukaryotic peptide is enhanced.

15

47. The method according to Claim 30 wherein said eukaryotic peptide other than a peptide of a plant plastid expressed in said plant plastid is folded with the correct number of disulfide bonds.

20

48. The method according to Claim 47 wherein said eukaryotic peptide is hGH

25

49. The method according to Claim 30 wherein said eukaryotic peptide other than a peptide of a plant plastid expressed in said transformed plant plastid is bioactive when isolated from said transformed plant plastid.

30

50. The method according to Claim 49 wherein said eukaryotic peptide is hGH.

20

51. A method for producing a non-methionine N-terminus protein in a plastid, wherein said method comprises:

transforming a plastid with a construct comprising, as operably joined components in the 5' to 3' direction of transcription

40

(a) a promoter functional in a plastid,
(b) a DNA sequence encoding a cleavable ubiquitin peptide,
(c) a DNA sequence encoding a protein of interest, and
(d) a transcription termination region; and

45

growing a plant cell comprising said transformed plastid under suitable conditions for expression of said protein of interest and said cleavable ubiquitin sequence in said plastid.

50

5 52. The method according to claim 51, wherein said construct further
comprises (e) at least two DNA regions of homology to the genome of said plastid.

10 53. The method according to claim 51, wherein said construct further
5 comprises (f) a gene encoding a selectable marker for selection of a plant cell comprising a
plastid expressing said marker.

15 54. The method according to claim 51, wherein said construct further
comprises (g) a ribosome binding site joined to said promoter (a).

10 55. The method according to claim 54, wherein said ribosome binding site is
20 derived from a leader sequence selected from the group consisting of a plastid leader
sequence, a bacterial leader sequence and a bacteriophage leader sequence.

15 56. The method according to claim 54, wherein said ribosome binding site is
selected from the group consisting of the binding site of the gene 10 leader and the
rbcLRBS site.

30 57. A plastid having a protein of interest produced according to the method of
20 claim 51.

35 58. The plastid according to claim 57 wherein said protein of interest comprises
at least about 1.0 % of total soluble protein in said plastid.

25 59. The plastid according to claim 58 wherein said protein of interest comprises
40 at least about 7.0% of total soluble protein in said plastid.

45 60. A plastid comprising a stably incorporated portion of the construct of claim
51 within its genome.

30 61. A plant cell comprising a plastid comprising a stably incorporated portion
50 of the construct of claim 51 within its genome.

5

62. A plant cell comprising a transformed plastid produced according to the method of claim 51.

10

5 63. A plant, plant seed or plant part comprising a plastid according to claim 57.

64. A plant, plant seed or plant part comprising a plastid according to claim 60.

15

65. A plant, plant seed or plant part comprising a plant cell according to claim

10 61.

20

66. A plant, plant seed or plant part comprising a plant cell according to claim

62.

25

15 67. The method according to claim 51 wherein said protein of interest is folded with the correct number of disulfide bonds.

30

68. The method according to claim 51 wherein said protein of interest is hGH.

20 69. The method according to claim 51 wherein said protein of interest is bioactive when isolated from said plant cell.

35

70. The method according to claim 69 wherein said protein of interest is hGH.

40

25 71. A plastid comprising:
a portion of a construct according to claim 51 stably integrated into its genome; and
a non-methionine N-terminus protein.

45

72. A plant cell comprising a plastid having:
a portion of a construct according to claim 51 stably integrated into its genome; and
a non-methionine N-terminus protein.

50

- 5 73. A method for producing a non-methionine N-terminus protein in a plastid,
wherein said method comprises:
transforming a plastid with a construct comprising, as operably joined components
in the 5' to 3' direction of transcription
10 5 (a) a promoter functional in a plastid,
 (b) a DNA sequence encoding a protein of interest, wherein said protein of interest
 is capable of being recognized by a plant cell Methionine Amino Peptidase, and
15 15 (c) a transcription termination region; and
growing a plant cell comprising said transformed plastid under suitable conditions
20 10 for expression of said protein of interest having a non-Methionine N-terminus.
- 20 74. The method according to Claim 73, further comprising the step of growing a
plant having said plant cell.
- 25 15 75. The method according to Claim 74, further comprising the step of harvesting
said plant and subjecting cells of said plant to means for substantially purifying said
protein of interest.
- 30 30 76. The method according to Claim 73, wherein said plant cell Methionine Amino
20 Peptidase cleaves the N-terminal methionine.
- 35 35 77. The method according to Claim 73, wherein the second amino acid of said
protein is selected from the group consisting of alanine, cysteine, glycine, proline, serine,
threonine and valine.
- 25 40 78. The method according to Claim 73, wherein the second amino acid of said
protein is alanine.

45

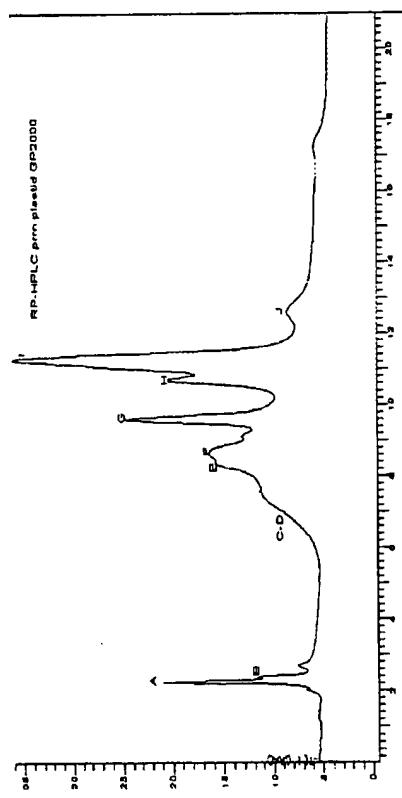
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5'-AAT TGT AGA AAT AAT TTT GTT TAA CTT GGA GAA GGA GAT ATA CC-3'
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FIGURE 1

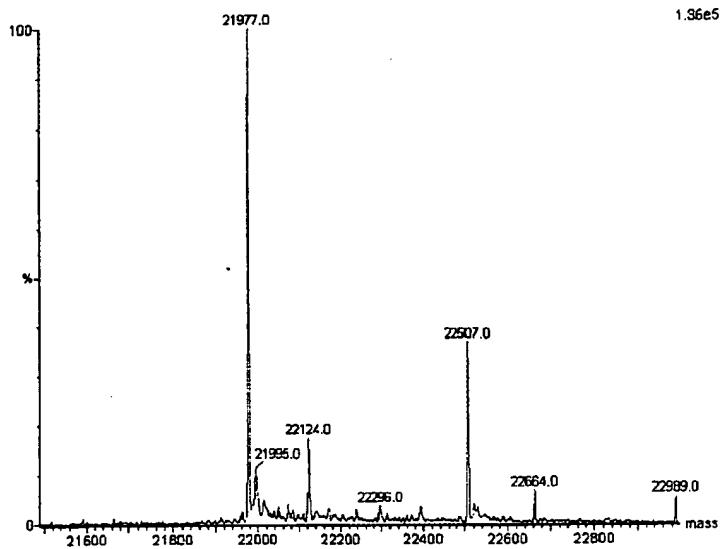
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Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-Lys-Arg-
Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-
Gly-Ala

FIGURE 2



RP-HPLC analysis shown above. Peak I (tallest peak) has the retention time expected for properly folded, native 22 kDa GP2000. This peak was collected and dried down in a Savant Speed-Vac for amino terminal sequencing and electrospray mass spectrometry.

FIGURE 3



Mass spectrometry of RP-HPLC peak I shown above. Note that there are 4 major protein species, of different molecular mass.

21977 kDa - This is the predicted mass of "des-Phe"-GP2000, ie. GP2000 with the predicted N-terminal Phe removed by over-cleavage of Ubiquitin protease.

****22124 kDa - This is the predicted mass of properly processed, correct amino acid sequence GP2000

22507 kDa - This is probably des-Phe-GP2000 modified during plant extraction procedures

22664 kDa - This is probably GP2000 modified during plant extraction procedures

Amino terminal sequencing was done by standard Edman degradation, and confirmed the N-terminal sequences discussed above.

Figure 4

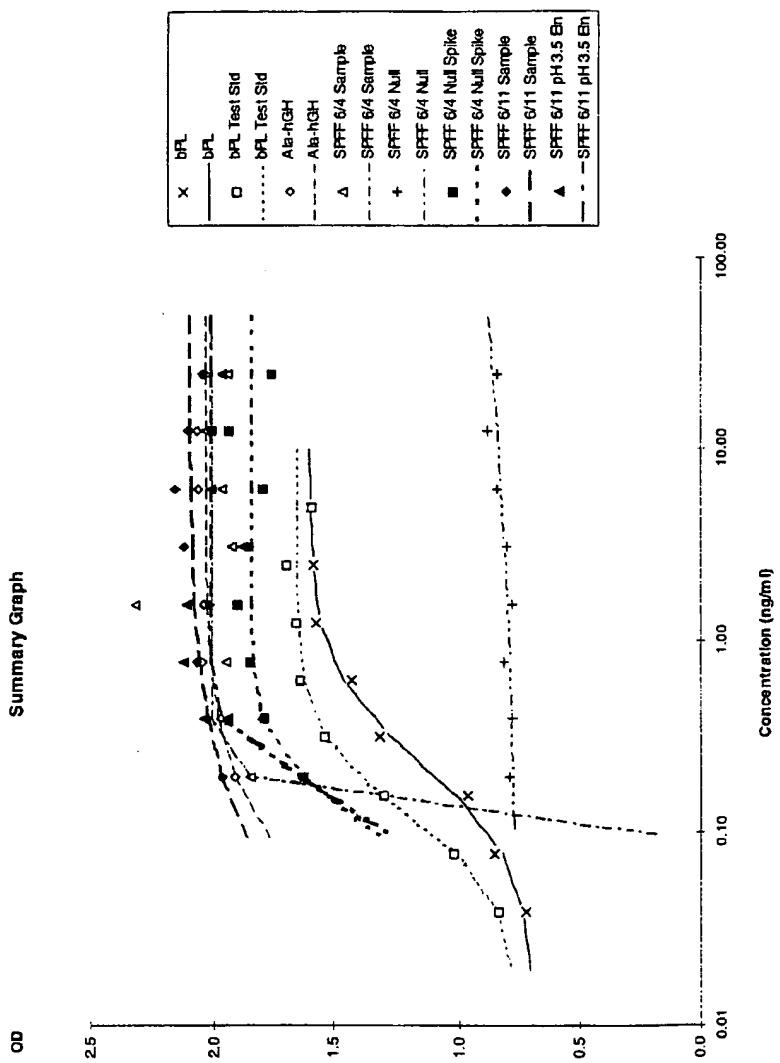


FIGURE 5

SEQUENCE LISTING

<110> Calgene LLC

<120> Expression of Eukaryotic Peptides in Plant Plastids

<130> 15346WO

<140> new application

<141> 1999-07-07

<150> 09/316847

<151> 1999-05-21

<150> 09/113244

<151> 1998-07-10

<160> 2

<170> PatentIn Ver. 2.1

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<213> from T7 bacteriophage from gene 10

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<211> 58

<212> PRT

<213> Homo sapiens

<400> 2

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1 5 10 15

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20 25 30

Phe Val Tyr Gly Gly Cys Arg Ala Lys Arg Asn Asn Phe Lys Ser Ala
35 40 45

Glu Asp Cys Met Arg Thr Cys Gly Gly Ala
50 55